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VERO CELLS

–Origin, Properties and Biomedical Applications

Department of Microbiology
School of Medicine Chiba University

The Twenty-fifth Anniversary of The Establishment

VERO CELLS

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Photographed by T. Nagano, Department of Anatomy School of Medicine Chiba University.

Preface

This year (1987) is the 25th anniversary of the isolation of Vero cells from African green monkey kidney tissue by Dr. Yosihiro Yasumura.

The Vero cell line was found to show high susceptibility to arboviruses and many other pathogenic viruses soon after its establishment, and scientists working on virus research quickly added Vero cells to their host cell collection. The range of application of the Vero cell line has been extended from basic virus research in laboratories to diagnostic practices in hospitals, epidemiological surveys and bacterial toxin assays. The cell line has been distributed to scientists throughout the world by reliable services of the American Type Culture Collection.

In recent years, the potentiality of Vero cells for vaccine production has been examined. The use of primary monkey kidney tissue for poliovirus vaccine production requires careful examinations of each lot of host cells for safety reasons. In addition, it has become extremely difficult to obtain primary African green monkey kidney tissue because of recent international agreements for the conservation of wild animals. Thus, several established African green monkey cell lines have so far been tested throughly as host cells for vaccine production. After extensive testing by the group of the Institut Mérieux in France, it has been demonstrated that low-passage-number Vero cells showed no tumorigenicity and contained no aberrant viruses and, furthermore, they sustained efficient proliferation of vaccine viruses. As the result, killed poliovirus vaccine using Vero cells has been available in France since 1984.

The Vero cell line is the fruit of Dr. Yasumura's toil. In 1962 he began work on the cultivation of cells from African green monkey kidney, which was supplied by the Chiba Serum Institute, a poliovirus vaccine maker, when he was associated with the Department of Bacteriology, School of Medicine, Chiba University. The isolation process was reported orally and recorded in the "Nihon Rinsho", a Japanese medical journal. However, it is not familiar to foreign investigators because the article has not been translated into English. On the occasion of the 25th anniversary of the establishment of the Vero cell line, we have compiled a book about Vero cells with the primary aim of introducing a translation of Dr. Yasumura's original report. A further major aim of this publication is to emphasize the great potential and versatility of the cell line by reproducing classical papers selected from the enormous number of Vero cell-related publications. It should be emphasized that these represent only a small part of the vast amount of significant scientific contributions involving the use of Vero cells in various biomedical fields by microbiologists and cell biologists throughout the world. The

book also describes the properties of low-passage-level Vero cells which have long been cryopreserved at Chiba University and the National Institute of Radiological Sciences, as well as some unpublished basic findings on the cells. The huge historical impact which Vero cells have had from academic and practical viewpoints makes it particularly important to insure that resources and research results should be properly maintained and widely disseminated.

Finally, we gratefully acknowledge the much needed editorial help given by Dr. Yosihiro Yasumura, Professor, Department of Microbiology, Dokkyo University School of Medicine, Dr. So Hashizume, Professor, Department of Pathobiology, School of Nursing, Chiba University and Dr. Shudo Yamazaki, Director, Central Virus Diagnostic Laboratory, National Institute of Health, Tokyo who had spent their youth together under Professor Yosio Kawakita at the Bacteriology Department. Thanks are particularly due to individual contributors for their original papers. We also wish to thank the authors and the publishers who kindly granted us permission for reproduction of their papers. In particularly, we wish to thank Jean Taylor-Wiedeman, M. D., Visiting Scientist, National Institute of Health, Tokyo for her valuable comments and advice for English usage throughout the preparation of the manuscript. We deeply acknowledge the Naito Fundation, Toray Science Foundation and Dainippon Pharmaceutical Co. for their generous financial supports of this book.

December, 1987

Bunsiti Simizu
Toyozo Terasima

Foreword

The Vero cell line has become a standard tool in virology, but few virologists, even those most familiar with the biological properties of these cells, know the history of their original isolation and culture. Dr. Yosihiro Yasumura's primary goal in 1962 was to find a homogeneous host cell line susceptible to Simian Virus 40 (SV40), which he wished to study as an oncogenic agent. The kidneys of the African green monkey (*Cercopithecus aethiops*) were severely affected by SV40 and seemed a logical starting point. Aware of the problem of adventitious agents and simian virus contaminants, Yasumura took great pains to obtain a clean cell line, including use of autoclavable media containing minimal serum. *C. aethiops* kidney cells were established in culture and successfully carried for many passages. They were shown to grow SV40 to high titer, with development of cytopathic effect, and under appropriate conditions of overlay, to provide a sensitive plaque assay. In 1963, Yasumura's results were published in a Japanese-language journal. This remarkable work has not been previously available in English, and the editors of this book provide a complete translation. This is an inherently interesting document of careful scientific methodology, but it also illustrates the serendipitous nature of science. For out of the logical approach to the problem of measuring one virus (SV40), grew many discoveries (some made by Dr. Simizu, Senior Editor of this book) about the usefulness of Vero cells for primary isolation, assay and growth of a wide variety of completely unrelated viruses. Included among the latter are some of the most unaccommodating agents responsible for human disease, such as the hantaviruses. The history of the arrival of Vero cells in the United States (in 1964) and their deployment in studies on arboviruses and hemorrhagic fever viruses is elegantly described in the chapter by Earley and Johnson.

Apart from the historical perspectives, original studies were undertaken, especially for this book, of the biological properties, cytogenetics, and isoenzyme of the lowest passage of Vero cells available. These results establish important baselines for comparative analyses of Vero sublines and clones in use today. The early Vero (passage 113) is preserved in The Japanese Cancer Research Resources Bank, whence it is available on request.

The application of Vero cells to the study of rubella, measles, herpes, and a variety of other viruses is documented in this book, and an excellent bibliography of relevant publications is included. The editors have performed a useful service, since the scientific community will continue to make use of Vero cells for analytic and determinative studies, as well as vaccine development. They have brought credit to Dr. Yasumura on the 25th anniversary of his

work.

The word "Vero" chosen by Dr. Yasumura, means "truth" in Esperanto. The etymology is of interest, since "Vero" is also an acronym formed from the constituent parts "Verda" (meaning "green" in Esperanto) and "Reno" (kidney): hence (African) green (monkey) kidney. Dr. Yasumura's use of Esperanto is appropriate to the universal application of his cell line.

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Isolation and Establishment of VERO Cells

Translated from Nihon Rinsho (in Japanese) *21*, 1201—1215, 1963

STUDIES ON SV40 IN TISSUE CULTURE — PRELIMINARY STEP FOR CANCER RESEARCH *in vitro*

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The preceding talk described a fine building already completed. To complete the metaphor, my subject may be likened to a building's foundation in the initial stages of construction. Since I have studied the polyoma virus for the last two years [1], SV40 will be discussed with reference to this problem. As Professor Yamamoto pointed out, Melnick [2] published a short review and introduced the term "papova virus" in "Science" this year. He states that SV40 may be classified into the same category as the polyoma virus.

CULTURE OF MONKEY KIDNEY CELLS

Since my involvement with the poliovirus vaccine, my attention has been focused on the wide range of viral growth in monkey cells. Their broad range of functions as host cells is a property which makes them quite useful to virologists. For this reason, the establishment of monkey kidney cells in culture has been my research target. Despite several attempts to isolate cells, however, I was unsuccessful. Since I have already dealt with the problem of the *Cercopithecus aethiops* kidney cell line [3] at the Tissue Culture Society, I will give only an outline here. Initial studies were performed on rhesus monkeys and crab-eating monkeys, but few cells were isolated. Last year, Associate Professor Katsuta of the University of Tokyo Institute for Infectious Diseases successfully isolated a kidney cell line from the crab-eating monkey [4]. In my experience, kidney cells from Japanese monkeys, crab-eating monkeys, and rhesus monkeys have all undergone vacuolar degeneration after one to one-and-a-half months of culture. All kidney cells from ordinary monkeys were lost during the course of culture. Because of

widespread infection by viruses generally called "simian viruses" [5], monkey cells cannot continue to proliferate over a prolonged period in culture, making isolation of a cell line difficult.

SV40 AND HOST CELLS

Sweet and Hilleman [6, 7] reported the discovery of the SV40, an organism widely distributed among rhesus monkeys. Eddy [8] subsequently reported implantation of rhesus monkey kidney cells in a hamster, with the subsequent induction of tumor formation. Recently, Eddy [9] and Girardi *et al.* [10] produced a tumor in the hamster with SV40 alone, thus demonstrating tumor formation by the virus itself.

I have decided to study this virus as an oncogenic agent. To perform the study, a host cell line was necessary. The most sensitive cells to SV40 infection were the kidney cells of the African *Cercopithecus aethiops* monkey. A cell line from these cells in culture would be ideal. Hsiung and Gaylord [11, 12] have reported studies in the Patas monkey. Attempts to isolate cell lines from a green monkey (*Cercopithecus aethiops*) represented an important priority in this field. As in my previous studies on polyomas [1, 13], the first target was to obtain the cells in as homogeneous a population as possible to facilitate subsequent manipulation and analysis. In addition, a host cell culture which was not contaminated by viruses was necessary. At least 52 viruses called simian viruses have been isolated from monkeys, including the SV40. In order to avoid contamination of the virus to be studied by other viruses retained in the cells, subculturing must first be conducted to confirm the absence of contamination. Among exogenous factors which influence culture purity, serum is probably the most frequently involved. Sera obtained from various animals such as calves and horses are usually used, but the constituents of such sera are largely unknown. To allow unknown components to be introduced into a culture is not desirable. Thus, the first thing to do is to remove serum from the culture medium and to try to obtain cell growth under protein-free conditions. Even if a protein-free medium is used, sterilization using a Seitz filter may allow filtrable microorganisms to pass. In order to guarantee sterility of the culture medium, sterilization of the medium by autoclaving [3, 13, 14] was attempted. It is of primary importance to produce a culture environment in which external contamination during the subculture process is very unlikely. By this method a clean cell line can be established. In such a system, only the study virus enters the cell. Naturally, nothing can be done if the virus to be inoculated is already contaminated. This initial work may be compared to the preliminary excavation for construction of a foundation, and much further effort will subsequently be required.

ESTABLISHMENT OF A GREEN MONKEY (*Cercopithecus aethiops*) KIDNEY CELL LINE IN CULTURE

A kidney cell line was established from the cells isolated from a kidney harvested from a *C. aethiops* monkey on March 27, 1962 as shown in Table 1. In the same table, previous attempts and failures to establish continuous kidney and embryo cell cultures are also shown with the number of subculture passages achieved. The coded date of culture initiation in the second column from the right uses the same code as that for dating canned food. For example, 8Y20

Table 1. Cultivation of monkey kidney tissues

No.	Monkey species	Beginning of culture	Passage no.
1	*M. fuscata yakui*	8Y20*	4
2	*M. cyclopis*	9304	2
3	*M. cyclopis*	9407	5
4	*M. mulatta*	9430	4
5	*M. irus*	9508	3
6	*M. cyclopis*	9616	5
7	*M. fuscata yakui*	9702	5
8	*M. irus*	0128	4
9	*M. irus*	0210	4
10	*M. irus*	0316	3
11	*M. irus* (embryo)	0526	7
12	*M. irus* (embryo)	0Y24	14
13	*M. irus* (embryo)	1414	8
14	*M. irus* (embryo)	1426	2
15	*M. irus* (embryo)	1714	6
16	*M. irus* (embryo)	1728	2
17	*C. aethiops*	1824	9
18	*C. aethiops*	2327	30**

*November (Y) 20, 1958.

**Vero cell line.

Table 2. Culture medium composition

	LH	LE	LE'	YLE' (g/l)
NaCl	8.00	6.80	7.20	7.20
KCl	0.40	0.40	0.40	0.40
$CaCl_2$	0.14	0.20	0.20	0.20 (0)
$MgSO_4 \cdot 7H_2O$	0.20	0.20	0.20	0.20
$NaH_2PO_4 \cdot 2H_2O$		0.16	0.16	0.16 (1.6)
$Na_2HPO_4 \cdot 2H_2O$	0.06			
KH_2PO_4	6.06			
Glucose	1.00	1.00	4.50	4.50
Phenol red	0.02	0.02	0.02	0.02
Lactalbumin hydrolysate	5.00	5.00,	5.00	5.00
Yeast extract				1.00
$NaHCO_3$	0.35	2.20	1.1~	1.1~
			2.2~	2.2~
			4.0*	4.0*

If PVP was used, the final concentration was 0.1%.

Antibiotics; penicillin 100 unit/ml, streptomycin 100 μg/ml.

* For maintenance.

( ) Suspension culture.

refers to November 20, 1958, 0128 to January 28, 1960 and 2327 to March 27, 1962. Although some cells went through 14 generations, many of them failed to proliferate, possibly because of some unknown latent virus, insufficient nutritional supply, or other undefined causes. An overwhelming infection by SV40 in the monkey specimen may have contributed most to the difficulty. In 1959, however, cells useful for the detection of SV40 had not yet been isolated.

Table 2 lists the various media used in cell culture. The medium currently used, indicated as YLE', has been previously described [1]. The salt solution forming the base is a modified Earle's medium, designated as E'. The modification consists of slightly higher sodium chloride and glucose contents. When the original Earle's solution was used, it was simply designated as E. I have specified the constituents of the medium in tabular form since various influences of medium constituents on cells have been noted. I want to emphasize the need for a clear definition of the specific medium used. Table 3 shows the formula for preparing the trypsin

Table 3. How to make "NACIKA-trypsin"*

$Na_3C_6H_5O_7 \cdot 2H_2O$	4.7 g (0.016 M)
KCl	0.75 g (0.01 M)
Trypsin (Bacto trypsin)	2.5 g
Distilled water	1000 ml
Adjust by NaOH to pH 7.6	

*According to McLeod and Whitmore

solution used for subcultures [15]. Instead of the currently used phosphate-buffered saline (PBS), a trypsin solution containing citric acid and potassium chloride was used because of its simplicity. This resulted in some difficulty. As long as the trypsin solution was used, consideration had to be given to the absence of exogenous filtrable factors in a culture, because the trypsin solution for subculture cannot be purified except by passage through a Seitz filter.

I will describe how subcultures were made of green monkey kidney cells, which led to the establishment of VERO cells. In most experiments, the cells suddenly lost their vigor in the fifth or sixth generation. When cells were taken from ordinary rhesus monkeys or crab-eating monkeys, vacuoles appeared, leading to various types of degeneration. In the case of the green monkey, subcultures could be continued at intervals of five days to one week without such degeneration. Since these cells exhibited no unusual morphology, one may assume that no infectious agent was present in the cells. With primary cells of the green monkey the subculture interval was sometimes as long as 29 days. This may be ascribed to certain physical characteristics of the glass used in the manufacture of the culture bottle, a topic which will be discussed later. Fig.1 illustrates the lineage of the various cultures from No.18 cells.

The cell line was so divided for security. One reason is that a cell line occasionally changes its properties during subculture [16-21]. Secondly, by dividing into as many different lines as possible, some may be used as a probe for media composition, especially changes in serum lot and concentration. After evaluating media components with probe cultures the changes

were extended to the parent culture which was cell line A in Fig. 1.

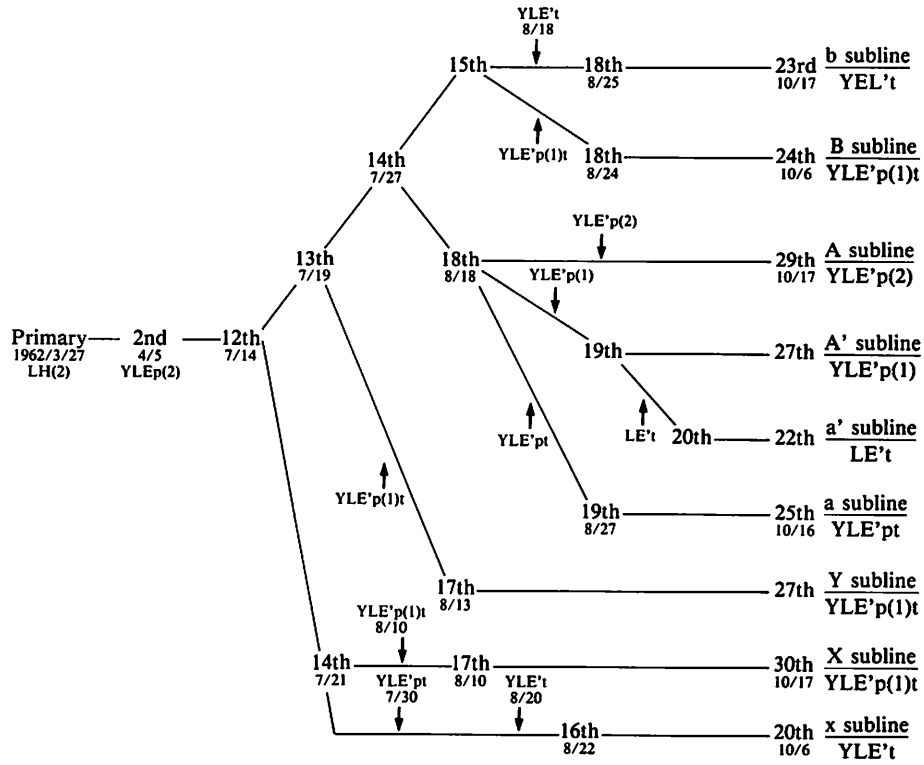

Fig. 1 History of the establishment of an African green monkey (*C. aethiops*) kidney cell line 'VERO', and the lineage. P indicates an addition of 0.1% PVP, t indicates autoclaved medium, and parentheses show the concentration % of calf serum used.

This cell line has been subcultured in 2% serum since culture initiation. Although subculture is very difficult at a 2% serum concentration, this represented a maximum serum concentration in our studies. The amount of serum in the culture system should be the minimum possible, because serum has been reported as a major factor in inducing cell change [22, 23]. In addition, when attempting to adapt the cells to a protein-free medium, sequential serum reduction was difficult starting from cells subcultured in 10 and 20% serum. For example, in our previous studies, it took up to 2 years to adapt "Sato carcinoma" cells to a protein-free medium [13]. The use of smaller amounts of serum in the initial culture makes adaptation to a protein-free environment possible over a much shorter period of time.

Subculture is often difficult with a 2% serum concentration. One reason for our success was the addition of 0.1 % polyvinylpyrrolidone (PVP)(indicated as p in the Figures) to the medium with 2% serum. PVP has been used as a synthetic oxygen carrier in blood transfusion practice. The Katsuta group at the Institute for Infectious Diseases has been working extensively on this idea in tissue culture and reported the feasibility of replacing more than 80% of the serum with PVP [4, 24-26]. This principle was successfully applied to monkey kidney cell culture.

One of the reasons for a 29-day subculture period between the 10th and 11th generations was thought to be the physical properties of the glass surface of the culture bottles used. These 2-ounce bottles (called Sani-Glas) were made in the United States [15, 27]. The other bottles were specially prepared by Kotobuki Special Glass, a glass producer in Japan, in order to economically produce plaque bottles in Japan at a time when a large number was needed for poliovirus plaque titration. Up to this time, cells have grown well in Kotobuki culture bottles. According to reports from the United States [15, 27] , Sani-Glas was used almost exclusively to assay poliovirus plaques, with favorable results. However, in our kidney cells subcultured at a 2% serum concentration, a subculture interval of 29 days resulted with Sani-Glas bottles. In new bottles used for the first time, washed only with a "soapless" soap, and cultured under identical conditions, only scattered cells grew on the Sani-Glas whereas confluent cultures were found on the Kotobuki glass. The sparse growth in the Sani-Glas bottles precluded further subculture. Two additional factors which may provide an insight into the mechanism of this confusing glass-specific cell growth are serum protein concentration and the inherent properties of the individual cell lines. Serum protein acts to facilitate cell adhesion to the glass surface. Especially in a protein-free medium, cells adhere poorly to glass, a problem which means that attention must be focused on the properties of individual glass surfaces [28]. However with Sani-Glas, the use of 10% serum in the medium did produce cell adherence indistinguishable from that obtained with the Kotobuki glass. As long as the Kotobuki glass is used, it is possible to grow green monkey kidney cell monolayers and to from viral plaques on it, even if a new bottle is used. Adequate monolayer growth naturally depends on the cell type as well. "Sato carcinoma" cells, for example, adhere quite well even in protein-free environments. "Fructose sarcoma" cells, on the other hand, are rather poorly adherent in a protein-free medium [13].

Despite various reports, especially those of Lieberman et al. [29] and Fisher et al. [30], concerning the serum dependence of cell adherence, no specific details elucidating the actual mechanism have been reported. Even with Sani-Glas, on which the kidney cells grow rather poorly, a massive initial application of the cells results in confluent growth, and at least two repetitions of a massive seeding of cells provides a surface favorable for growth of the cells. According to Weiss [31], after cell adherence to the glass surface, part of the cell surface components remains on the glass surface and, furthermore, the glass surface undergoes alteration by the cells. This theory, which postulates an apparent physical "conditioning" of the glass surface, is well supported by our culture results. In a bottle in which cells have grown several times, subsequent cell seeding produces a beautiful monolayer growth which forms over the entire surface. However, in a new bottle, no growth will occur under identical culture conditions.

The typical appearance of subcultured green monkey kidney cells is illustrated in Fig. 2. These cells commonly have two nucleoli, but occasionally may have three. While these cells are mostly spindle-shaped, they do not necessarily represent a fibroblast lineage. The shapes of the cells may change if the amount of serum is increased or if the protein is removed completely. In kidney cells from other animal sources cultured *in vitro*, spindle-shaped cells appeared from peri-glomerular cells whereas polygonal cells appeared from tubular cells according to one report [32]. The origin of these cells is not fully understood. Fig. 3 illustrates the adjoining or overlapping of cells after about 14 days of culture.

Since efforts have been turned to the induction of cell transformation in this cell line

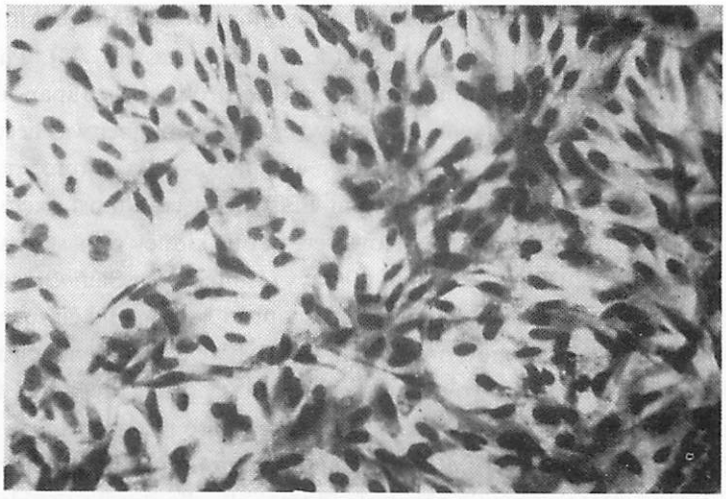

Fig. 2 Vero cells, *A* subline after 6 days of cultivation with YLE'p(2) medium. Magnification ×400

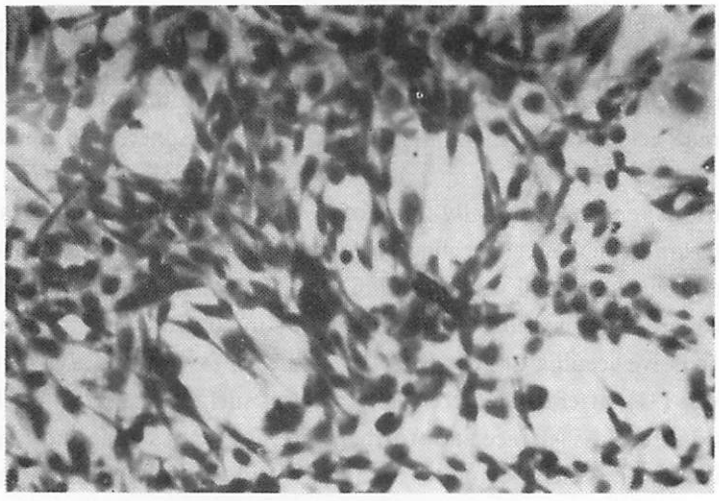

Fig. 3 Vero cells, *A* subline after 12 days of cultivation with the same medium as used in Fig. 2. ×400

by SV40, as has been done with the polyoma virus [33-35], careful observation of control cultures was the first requirement. The parent strain A grown in 2% serum presents with a net-like structure. When the serum concentration is higher than 2%, an even greater degree of overlapping occurs. Furthermore, on continuation of the culture, cells in the control culture commonly generate an overlapping pattern as well. To study these long-term growth characteristics, for example, the cells from the control cultures were not discarded, but were maintained in culture for about four months in serum-free, autoclaved LE' solution. After one to

two months, islands of epithelioid cells appeared within a sheet of spindle-shaped cells (Fig. 4). This long-term cell growth pattern may be related to a report by Shein [36] regarding the

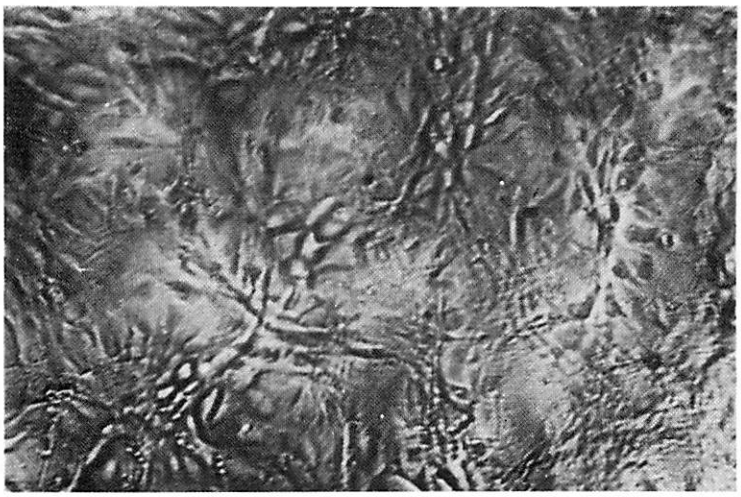

Fig. 4 Vero cells, *A* subline at the 15th passage level. The cells were maintained for 3 months with LE't medium. ×400

transformation of human kidney cells in culture with SV40. Within the paper, it was mentioned that the next step would be to demonstrate virus release from these apparently transformed cells. It may be a bit risky to identify cell transformation based on morphologic appearance alone, as epithelioid cells of this same morphology appear in control cultures of green monkey kidney cells. When a culture is inoculated with the virus, observation usually lasts at most two or three weeks, after which time the culture is discarded. The control cell cultures are usually discarded after confirming the absence of morphologic alteration. However, on two occasions in our laboratory, control cell cultures were serendipitously maintained on LE' solution for long periods. Cells of a previously unreported nature appeared, possibly because the yeast extract was omitted. This "transformed" morphology has been observed in the un-inoculated control culture, although Shein did not report cells with such an appearance in his control group.

Thus, although the epithelioid cells which appeared following virus inoculation were reported as transformed cells, there is a need for confirmation independent of morphology. There are many ways to confirm transformation, such as virus recovery from the cells, or tumor formation after reimplantation into an animal host. Morphology may not be an adequate sole marker of transformation, and the development of an independent confirmatory test is necessary.

In this context, the use of filtrable agent-free media is critically important. Autoclave sterilization was frequently used in these cell cultures. Preparation of YLE' medium is shown as an example of medium sterilization (Table 4). While cell growth characteristics in any culture are cell-line dependent, fructose sarcoma and Sato carcinoma cells grew somewhat less actively in autoclaved serum-free YLE' as compared to Seitz-filtered YLE'. However, 60 to

70 generations of Fructose sarcoma and Sato carcinoma cells have already proliferated in an autoclaved medium [13].

Table. 4 Preparation of autoclaved YLE culture medium

Stock solution (Store at 5°C)

Stock A; 10Xconc.		Stock B; 10Xconc.	
NaCl	71.8 g/L	$CaCl_2$;	2.0 g/L
KCl	4.0	$MgSO_4 \cdot 7H_2O$	2.0
NaH_2PO_4	1.25		
Glucose	45.0		
Phenol red	0.2		

Mixture of components

A	B	L*	Y**	PVP	Total volume
100 ml	100 ml	5 g	1 g	1 g	1,000 ml

* Lactalbumin (N.B.C).

** Yeast extract (Difco).

Adjustment and Autoclaving

Step 1. Combine A, B, L, Y, PVP in the proportions listed above, and adjust to 1,000 ml in volume with distilled water.

Step 2. Dispense 7.5% $NaHCO_3$ solution in a prescription bottle and stopper tightly.

Step 3. Autoclave step 1 and step 2 solutions separately at 115°C at 10 pounds per square inch for 10 min.

Step 4. Add 15 ml of step 2 solution to 1,000 ml of step 1 solution (1.1 g $NaHCO_3$/L) after autoclaving both solutions.

The application of autoclave sterilization to various media can lead to difficulty. For example, PVP withstands autoclaving, but cavalier application of this method to Earle's solution containing both lactalbumin and yeast extract may be rather inadvisable. No such application of autoclaving to Earle's solution has been reported, although it has been applied to Hanks' solution. Earle's solution was not originally designed for autoclaving, requiring the presence of CO_2. However, autoclaved medium does appear to be sufficient for practical use in cell cultures and the most important aspect is that autoclaving is very efficient for the removal of exogenous contaminants. Most of the cell lines in this laboratory are now cultured in autoclaved medium. One cannot predict the long-term characteristics of this medium, but the cells are now proliferating, permitting at least 12 to 18 months of constructive use.

Fig. 5 illustrates cells subcultured in serum-free autoclaved medium containing PVP. When new glass containers are used, it is impossible to grow a good monolayer in a protein-free medium. As presented to the Tissue Culture Society [3], a special technique was used. Cover glasses which had previously been plated with cells were cleaned and reused. The recovered cover slides were used to grow the cells in a monolayer in protein-free medium. Similarly, the bottles were repeatedly seeded and the history was inscribed on the outside of the bottle. In this way, favorable growth was achieved even in protein-free medium.

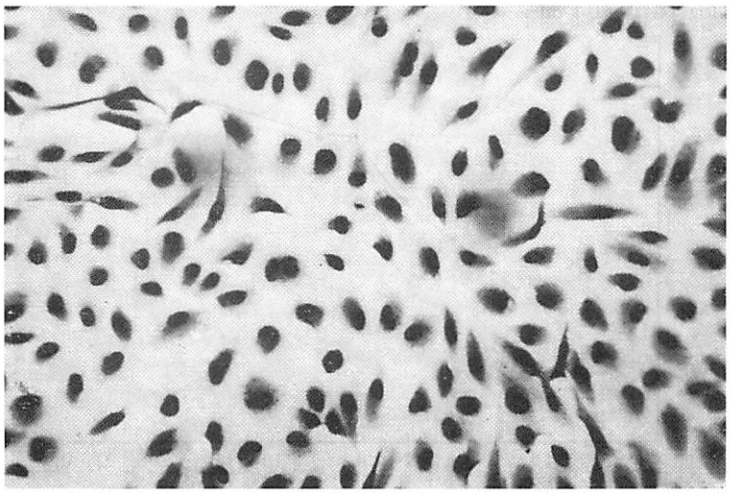

Fig. 5 Vero cells, *a* subline after 6 days of cultivation with YLE'pt medium. ×400

Another difficulty with protein-free medium is the inability to generate a meaningful cell growth curve. At present, variations of the growth curve are encountered depending on the bottle or test tube used. After data collection from a bottle, it may be possible to draw a growth curve for a bottle the characteristics of which are well known. Cells in YLE alone without PVP grow somewhat less rapidly.

RESPONSE TO SV40

Fig. 6 illustrates the viral growth curve of SV40 in Vero cells. Observation was usually discontinued on the 14th day for titration. After a peak on the 12th day, no remarkable change of virus yield occurred, even up to the 21st day.

Cells of subcultured lines occasionally undergo spontaneous alterations. For example, during work with the Japanese encephalitis virus using a hamster kidney cell line [1] and testicular cell line [37], there was a sudden drop in the cells' sensitivity to the virus [37]. The present Vero cells form a monolayer in 2% serum only for about five days. On the fifth day, passage is made over an area twice as wide as before, according to the method of Hayflick [38]. Diploid cells should not be multiplied too much, in order to retain the diploid state. With excessive proliferation, the rate of spontaneous variation of the cells rises. Only the standard subline A was maintained in culture medium passed through a Seitz filter. All other sublines were switched to an autoclaved medium. On the 5th to 7th day at a serum concentration of 1% and on the 10th to 14th day in a protein-free system, the cell sheet fills the bottle. The number of cultures doubled after every subculturing. The sensitivity of the cells to SV40 did not change in the 6th, 15th, 1 6th, 22nd or 25th generations. When cells are allowed to proliferate further, this sensitivity may occasionally change. In the *C. aethiops* kidney cell line reported by Dr. Tagaya of the National Institute of Health [39] at the Tissue Culture Society Meeting, the sensitivity to SV40 apparently decreased. According to the report by Dr. Ito [40] at the Virus So-

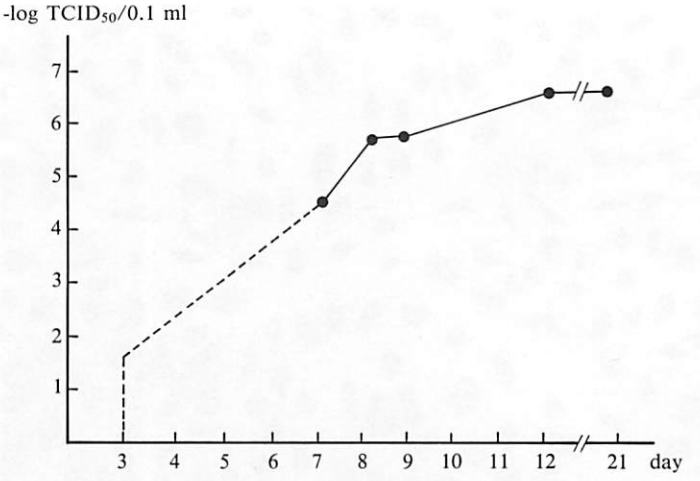

Fig. 6 Virus yield of SV40 in Vero cells. Culture fluid containing viruses was harvested 3 to 12 and 21 days after virus inoculation. The culture fluid was serially diluted 10-fold and each diluted fluid was inoculated into 5 Vero cell tubes (0.1 ml per tube) at each dilution for virus titration. Vero cells were maintained with LE't medium.

ciety Meeting, the fall amounted to about 2 logs. Although details of the culture history were not reported, some cells may behave in this fashion. Therefore slowing the speed of proliferation may be appropriate.

In Vero cells, SV40 proliferates to a level of about $10^{7.5}$/ml, which provides abundant opportunity for experimentation. In BS-C-1 cells [41] (isolated from C. aethiops by the Division of Biological Standards, NIH), SV40 also increased to approximately $10^{7.5}$/ml.

Fig. 7 shows the control cells in inoculation experiments with SV40. These cells appear

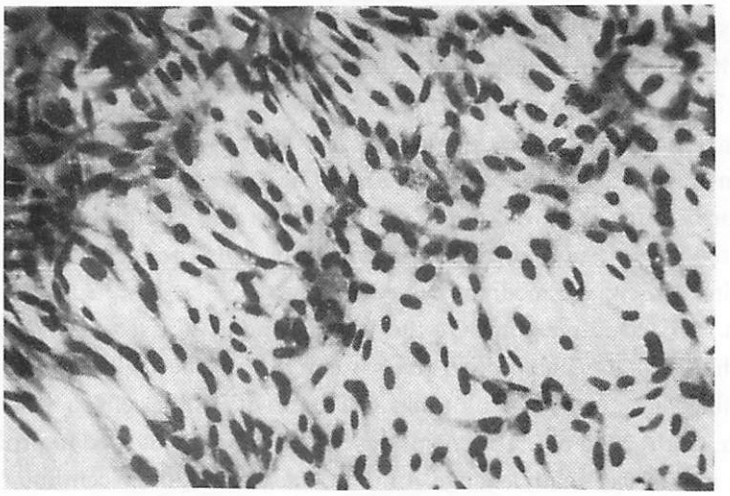

Fig. 7 Vero cells, *A* subline, uninoculated control cells maintained with LE't medium for 7 days after mock infection. ×400

quite slender since they were transferred from serum-containing medium to serum-free LE' medium for maintenance. The monolayer, however, is not severely disorganized. Fig. 8a shows

Fig. 8a SV40-inoculated Vero cells *A* subline 7 days after infection. ×400

the culture on the 7th day after inoculation with SV40 [3]. By this time, the maintenance medium consists of LE' alone. Around the 7th day, the surrounding cells have a poor appearance, with many cells in the center exhibiting necrosis. After about 14 days (Fig. 8b), most of these

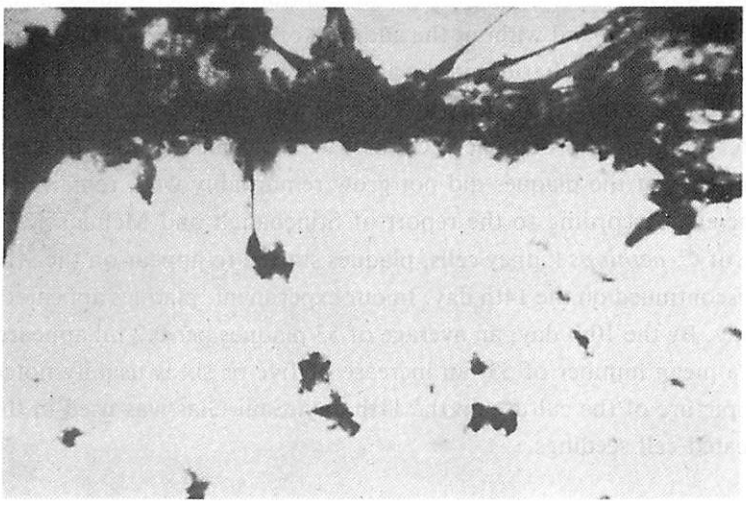

Fig. 8b Vero cells 12 days after infection. ×400

cells slough off. It is quite difficult to keep these cells alive for long periods, as with polyoma-inoculated cells [1]. It may be possible to produce transformed cells from among the surviving cells, but attempts have so far been unsuccessful. In the cases of polyoma, surviving cells remain, whereas after inoculation with SV40, a whole sheet of cells may slough, making it difficult to keep surviving cells growing.

PLAQUES OF SV40

Attention was then turned to the formation of plaques to test the utility of these cells for virus quantification. Serum-free LE' was used for this purpose. Four grams of $NaHCO_3$ per liter was added, according to Melnick [27]. Monkey cells are certainly more stable at a higher pH. A concentration of neutral red (NR) of 1:20,000 or 1:30,000 is too low. The formula for the agar overlay medium is given in Table 5. Reports on plaque formation using pro-

Table 5. Preparation of agar overlay medium

First overlay medium		
a*	Agar (Difco Bacto-Agar)	1.5 g
	10 × conc LE or LE'	10 ml
	dist. H_2O	83 ml
b	7.5% $NaHCO_3$	5.4 ml
Autoclave solutions a and b separately at 10 pounds per square inch for 10 minutes, and then mix well.		
Second overlay medium (5 days after 1st overlay)		
Same as the 1st overlay medium, and add phenol red 1:20,000 in final concentration.		

*In the case of addition of protamine sulfate, add 10 ml of 0.4% protamine sulfate solution to solution *a* (adjusted with dist. H_2O to 73 ml) and then autoclave.

tamine sulfate [42, 43] have recently appeared. In addition to other studies, a comparison was made between cultures with and without the addition of protamine sulfate. When the overlay solution is acidic, acid polysaccharides in the agar may be toxic to the cells [44]. Added protamine, however, decreases the cell toxicity by binding with the polysaccharides. Large plaques are obtained after addition of protamine in cultures with encephalomyocarditis virus [42]. SV40 was similarly tested, but the plaques did not grow remarkably well, remaining between one and two millimeters. According to the report of Stinebaugh and Melnick [27], who used a primary culture of *C. aethiops* kidney cells, plaques started to appear on the 9th day, and observation was discontinued on the 14th day. In our experiment, plaques appeared from around the 7th or 8th day. By the 10th day, an average of 53 plaques per 0.2 ml appeared at 10^5 dilution. Based on a mean number of 53, an increase of five or six is usually noted by the 14th day. Fig. 9 is a picture of the culture on the 11th day. Sani-Glas was used in this experiment after three repeated cell seedings.

ATTEMPTS AT CELL SUSPENSION CULTURE

An attempt to raise the virus yield was the next task. As long as the cells remain in a monolayer, 10^9/ml would be the maximum number of rapidly proliferating viruses even under optimum conditions. It is extremely difficult to increase the yield to more than 10^9. An increase in the host cell number, then, is the only solution. Even when monolayer cell numbers

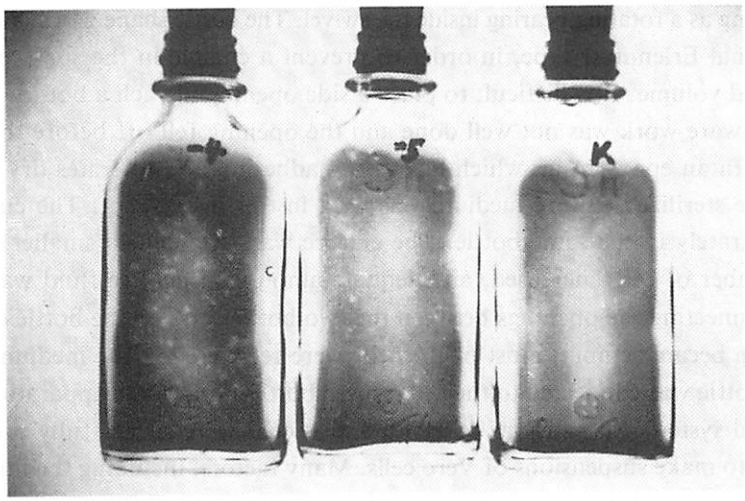

Fig. 9 Plaques of SV40, stained at 6 days after the second agar overlay. Inoculum was 0.2 ml per bottle.

are increased, for example, with HeLa cells, the limit is approximately 1.5 million per ml at best. The Vero cells may reach 800,000 to 1 million per ml, even with proliferation to the point of overlapping. In theory the virus yield may reach 10^8 to 10^9 when the burst size is 100 to 1000. Higher values are hard to achieve with this technique. A suspension culture of cells is one possible approach to increasing the cell number. When the rate of proliferation of the cells rises and mutation to a reduced sensitivity occurs, they may no longer have good utility as a host. This should be checked frequently. In the incubation room of our department (Fig. 10) suspension cultures are produced by utilizing the spinner culture method frequently employed by McLimans et al. [45]. This illustrated stirrer is a tentative model. A magnet bar in the bottle was installed according to Salzman [46], using a swivel called a "back twister" by fishing tackle handlers. The Teflon-covered magnet bar was hung with a stainless steel wire

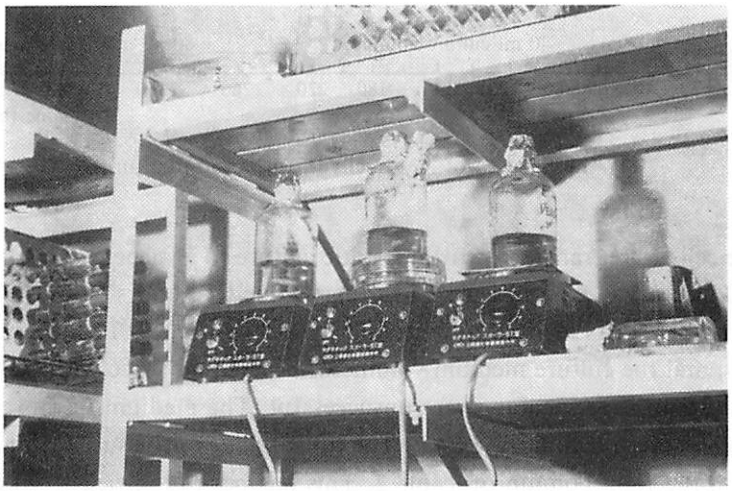

Fig. 10 A photo of Vero cell suspension cultures in a warm room.

from a ball acting as a rotating bearing inside the swivel. The bottle shape was a straight cylinder, and not the usual Erlenmyer type, in order to prevent a change in the surface area with an increase of fluid volume. It is difficult to place a side opening in such a bottle. In this experiment, the glassware work was not well done and the opening fell off before the experiment. It was fixed with an epoxy resin, which has strong adhesion and tolerates dry heat sterilization. Autoclave sterilized culture medium was used in this instrument. The culture medium was made separately in the same bottle. The culture was started at a smaller fluid volume, but as the number of cells increased, an adequate amount of culture fluid was added via a rubber tube connecting the openings between the two bottles. When the bottle containing the culture medium became empty, most of the cells were recovered in the medium bottle and a new medium bottle was connected to the cell growth bottle. The whole apparatus can be operated as a closed system. Suspensions of HeLa and L cells were successfully prepared, but it is still difficult to make suspensions of Vero cells. Many factors, including the number of revolutions of the stirrer and the inoculum size of the cell apparently influence the degree of success. Since suspensions usually permit rapid cell growth, Earle's solution is used. In the United States, CO_2 is initially used to balance the pH. Since this complicates the procedure, we are attempting to bypass this step. A proliferation curve at this stage appears in Fig. 11.

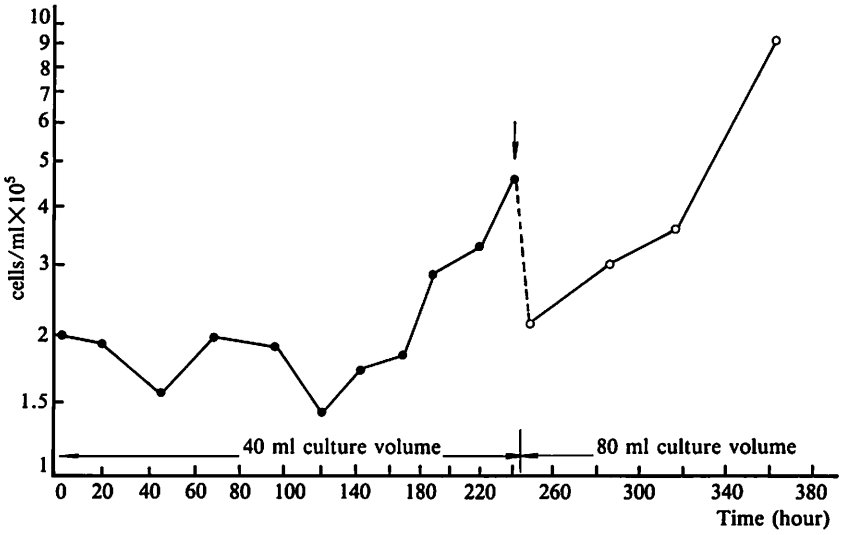

Fig. 11 Growth of Vero cells in suspension cultures.

When the cells were transferred from a static culture to a suspension culture, a lag period of up to 120 hours was experienced. This is partly due to 4 hours' standstill of the stirrer. Once the cell number starts to rise, however the growth continues. The cell number doubled by the 10th day, and the culture medium volume was increased twice with addition of 40 ml. Attempts to use serum-free medium were unsuccessful. The medium in this case consists of YLE' containing 1% serum and 0.1% PVP. The constituents of the suspension fluid are given in parenthesis in the right column of Table 2. This has been modified from the formula by Salzman [46]. The only modification was removal of calcium chloride and a 10-fold increase

in the phosphate content. In Eagle's laboratory, L and HeLa cells were easily grown in a spinner culture. The growth of Vero cells was quite poor in the beginning. The initial proliferation was poorer than in static cultures, but once the rise began, it continued. The generation time at this stage is 30 to 40 hours at best, but this is not constant. In the medium containing 1% serum, cells are vulnerable and easily destroyed when the stirrer is used. Staining with trypan blue or erythrosin B reveals about 15% cell loss. The efficiency is thus much lower than the reported 2-5% cell loss in suspension culture. In addition to 1% serum, other buffering materials such as methyl cellulose [45, 47] may be added in future experiments.

CONCLUSION

Cercopithecus aethiops kidney cells were subcultured continuously in autoclaved medium with or without serum and found to sustain proliferation of SV40. Quantitative assay of the virus was successful by plaque formation on the subcultured *Cercopithecus* kidney cells (Vero). The virus yield may be increased by growing cells in suspension culture. With a reliable high yield method for tumor virus proliferation, this research provides a foundation upon which further studies of tumors may be based.

■ **Note Added in 1987**

VERO/vé:ro/*n.* [Esperanto *vero* truth < Esperanto *verda(-simia) reno* green (monkey's) kidney]

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2.

History of VERO Cells in Japan and the United States

HISTORY OF VERO CELLS IN JAPAN

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Until 1964, Vero cells had long been passaged in growth medium supplemented with a low concentration of calf serum in the Department of Bacteriology (Prof. Y. Kawakita), Chiba University School of Medicine. The growth medium used during that period was 2% calf serum-fortified YLEP, which was composed of yeast extract (Difco), lactalbumin hydrolysate (Nutritional Biochem. Co.) dissolved in Earle's saline and 0.1% polyvinylpyrrolidone, as described in the original report by Yasumura and Kawakita(1).

From 1964 onward, N16SF (2) or F10 HI (3) medium supplemented with calf serum was used for passage generations 103 to 109. The culture was then kept frozen in solid CO_2 and later in liquid nitrogen in the Cell Culture Research Section of the National Institute of Radiological Sciences (NIRS), Chiba, until 1985. The history of this cell line is summarized in Table 1. In 1985, all frozen stocks were transferred to the Department of Microbiology (former Department of Bacteriology; Prof. B. Simizu),Chiba University School of Medicine. The cells were thawed, allowed to proliferate in 10% fetal calf serum-MEM, then frozen again on Aug. 21, 1985, and served as reference samples thereafter.

Table 1. History of Vero Cells at NIRS

Passage No.* of Culture	Growth Medium	Serum	Remarks
early generations	refer to Yasumura's report (1)		
Vero (102p)	YLEP	2% calf	a glass bottle culture was transferred to NIRS in Nov., 1964.
(103p)	N16SF	2% calf	grown at NIRS.
(104p)	N16SF	2% calf	
(105p)	N16SF	2% calf	
(106p)	N16SF	2% calf	
(107p)	N16SF	2% calf	frozen** at NIRS on Nov. 26, 1964.
(108p)	F10HI	2% calf	
(109p)	F10HI	2% calf	frozen** at NIRS on July 30, 1965. frozen stocks*** were moved to Chiba Univ. in July, 1985.
(110p)	MEM	10% fetal calf	
(111p)	MEM	10% fetal calf	frozen*** at Chiba Univ. on Aug. 21, 1985.

*Passage number is given in parentheses.

**Frozen in solid CO_2; 10% DMSO was consistently added as a cryoprotectant.

***Frozen in liquid N_2.

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CELL LINE VERO DEPOSITED TO JAPANESE CANCER RESEARCH RESOURCES BANK

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Vero, a cell line established from the kidney of African green monkey, was submitted to Japanese Cancer Research Resources Bank(JCRB)-Cell on June 5, 1987. Two frozen ampoules of passage number 111, prepared on Aug. 21, 1985 by Dr. Bunsiti Simizu, School of Medicine Chiba University, which is the youngest culture available now, were deposited to JCRB. As soon as the cells were received, cultures were established in antibiotic-free medium. The cultures were grown through three passages, after which contamination check for bacteria, fungi and mycoplasmas were done. The origin of the cells was verified by isoenzyme analysis.

The method for the preparation of cell suspensions, for bacterial, fungal and mycoplasmal contamination tests was as follows. Cells were quickly thawed in 37°C water bath and were washed with fresh minimal essential medium (MEM) containing 5% fetal calf serum without antibiotics. The cells were inoculated to culture dishes (diameter 10 cm) and cultured in the same medium at 37°C in a humidified CO_2 gas incubator. The cultivation was continued for two weeks including one passage after which the cells were transferred to glass ampoules, frozen in a programmable freezer (Nihon Freeze TNP80), and stored in a liquid nitrogen reservoir. A part of the culture was withdrawn before freezing and cultured for additional two passages for the contamination tests and the isoenzyme analysis. For both assays the protocol established in American Type Culture Collection was applied [1]. No contamination by viable bacteria, fungi or mycoplasma was observed in the Vero sample.

Isoenzyme analysis was performed by using a kit obtained from Corning Co. Enzyme extraction was done as instructed by the manufacturer. One microliter of the cell extract was loaded on agarose gel plates with control cell extracts, i.e. Vero, (ATCC CCL 81), HeLa (ATCC CCL 2) and NCTC clone 929 (ATCC CCL 1). The agarose gel plates were stained with seven different substrates. The results of the analysis (Fig. 1) showed that all bands observed for the Vero deposited to JCRB consistently moved with Vero obtained from ATCC (CCL 81).

The Vero prepared in JCRB cell bank was preserved under the code number JCRB0111, passage number 113, which will be distributed on request.

Reference

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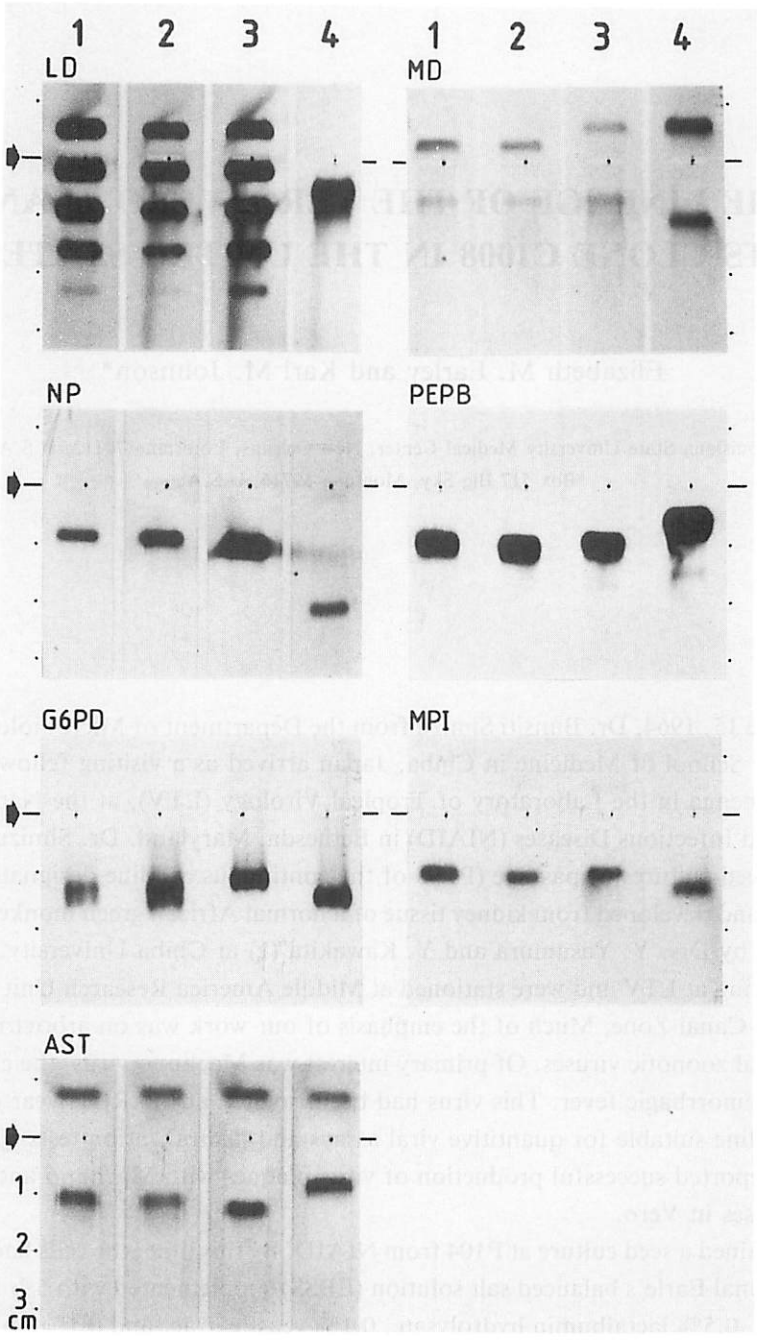

Fig. 1 Zymogram for Vero cells. Lane 1: Vero (JCRB0111), Lane 2: Vero (CCL 81), Lane 3: HeLa (CCL 2), Lane 4: NCTC clone 929 (ATCC CCL 1). Abbreviations are as follows. LD: Lactate dehydrogenase (EC 1.1.1.27), NP: Purine nucleoside phosphorylase (EC 2.4.2.1), G6PD: Glucose-6-phosphate dehydrogenase (EC 1.1.1.49), AST: Aspartate aminotransferase (EC 2.6.1.1), MD: Malate dehydrogenase (EC 1.1.1.37), PEP B: Peptidase B (EC 3.4.11.4), MPI: Mannose phosphate isomerase (EC 5.3.1.8). Arrows indicate origins.

THE LINEAGE OF THE VERO, VERO 76 AND ITS CLONE C1008 IN THE UNITED STATES

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On June 15, 1964, Dr. Bunsiti Simizu from the Department of Microbiology of the Chiba University School of Medicine in Chiba, Japan arrived as a visiting fellow to work with Dr. Ned Wiebenga in the Laboratory of Tropical Virology (LTV), at the National Institute of Allergy and Infectious Diseases (NIAID) in Bethesda, Maryland. Dr. Simizu brought with him several seed cultures of passage (P) 93 of the continuous cell line designated Vero. Vero was initiated and developed from kidney tissue of a normal African green monkey, *Cercopithecus aethiops,* by Drs. Y. Yasumura and Y. Kawakita (1) at Chiba University. At that time, we were working at LTV and were stationed at Middle America Research Unit (MARU) Balboa, Panama Canal Zone. Much of the emphasis of our work was on arboviruses and other related tropical zoonotic viruses. Of primary interest was Machupo virus, the causative agent of Bolivian hemorrhagic fever. This virus had been isolated at MARU a year earlier and we sought a cell line suitable for quantitive viral assays and neutralization tests. Drs. Wiebenga and Simizu reported successful production of virus plaques with Machupo and several other zoonotic viruses in Vero.

We obtained a seed culture at P104 from NIAID. By this time, the cells had been weaned from the original Earle's balanced salt solution (EBSS) supplemented with 5% heat inactivated calf serum, 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 0.1% polyvinylpyrrolidone to Medium 199 supplemented with 5% fetal bovine serum (FBS) or to Eagle's Minimum Essential Medium (EMEM) in Earle's balanced salt solution (EBSS) supplemented with IX non-essential amino acids (NEAA) and 5% FBS. The Vero cell line was submitted by Drs. Hann (formerly at NIAID) and Rhim (LTV, NIAID) to the American Type Culture Collection (ATCC) at P113. ATCC designated Vero as ATCC CCL 81. ATCC expanded the 25 cm^2 flask and prepared their stock seed pool at P121. All ampules of Vero at ATCC are from the expansion of the P121 pool. The ATCC shipping record of instructions states that Vero CCL 81 is grown on Medium 199 with 5% FBS but notes that cells also grow adequately on EMEM

with NEAA and 5% FBS.

In Panama we received a 25 cm^2 glass bottle of Vero from Dr. Wiebenga on 9/13/64. It was the fourth passage from his P100 frozen stock. This flask was subcultured for two passages on Medium 199 with 5% FBS and frozen at P106, MARU pool #41, on 9/22/64. A second pool was prepared from Pool #41, P106, and frozen as P124 pool #76, on September 14, 1967 by Ms. Gladys Oro. Our investigation of this line for plaquing and neutralization tests of arboviruses using small well plastic trays gave very good results (Earley, Peralta and Johnson, 1967(2). Previously, arbovirus neutralization tests were done in mice, and were expensive laborious tasks. With the susceptible continuous Vero cell line and the assay in the small well trays, a routine cell culture system for doing plaque neutralization tests of sera for titers and for direct isolation and identification of arboviruses became a reality as well as an economical accomplishment. We were also able to plaque the new virus isolate from Bolivia classified as an arenavirus.

Dr. Earley left MARU to attend graduate school and after receiving her Ph.D. in 1971, was appointed to the position of Chief of the Cytogenetics Section, Division of Pathology at the Bureau of Biologics, Food and Drug Administration in Bethesda, Maryland.

Dr. Johnson moved to the Communicable Disease Center (CDC) in Atlanta, Georgia in 1975 and he brought ampules of Vero from pool #76 (P124). Thus, the name Vero 76 was recorded. These ampules were expanded four passages at the CDC Special Pathogens Tissue Culture Laboratory and frozen at P128, 6/24/77. Vero 76 was switched from Medium 199 to EMEM with IX NEAA and 5% fetal bovine serum.

Although Vero 76 supports replication of, and plaque production by, a wide variety of viruses, this continuous epithelial cell line is noncontact inhibited and has a high rate of metabolism which limits plaque assays to about 7-10 days. Vero 76 cells when used as liquid phase cultures for viral isolation or titration work, require fluid changes twice weekly.

This latter problem, in particular, was of great importance at CDC in connection with quantitative clinical virological studies on Lassa fever in Sierra Leone, West Africa. The liquid phase Vero cultures were more sensitive and gave more reproducible results than plaque assays for specimens from patients and wild rodents. However, such cultures were enormously expensive to maintain because of the working conditions imposed by the need to restrict work to a Maximum Containment Laboratory (P-4). In a search for a Vero subline which was both Lassa virus sensitive and could be maintained without fluid change for 7-10 days, Dr. Paul Price established seven clones which displayed four distinct morphological growth patterns. Several of these clones were equally sensitive to unpassaged Lassa virus but clone E6 was chosen because it could easily be maintained 7-10 days without changing medium. This clone, designated C1008, was expanded and frozen at P4.

Dr. Johnson sent a flask of Vero 76 (P139) and Vero C1008 (P6) to the Bureau of Biologics in 1980 for verification by cytogenetic analyses that both lines were of African green monkey origin. Slides of metaphase chromosomes were prepared from each culture and stained with Giemsa. The normal diploid or 2N number of chromosomes of the African green monkey is 60. There is one distinctive pair of medium size subtelocentric chromosomes easily recognized by a prominent secondary constriction just below the centromere that is a diagnostic marker for the African green monkey karyotype. The microscopic examination of the metaphase

chromosomes showed the presence of the two diagnostic marker chromosomes in each cell of Vero 76 and its Clone C1008. Chromosome counts of cells revealed a hypodiploid modal number of 58 in each line. Slides were also processed and stained to reveal C-bands. Typically, all chromosomes of the African green monkey have a C-band at each centromere. The metaphase chromosomes of Vero 76 exhibited this pattern. Vero C1008 also had this C-band pattern in each chromosome but in nearly 80% of the cells there was a medium size telocentric chromosome with an additional interstitial C-band.

Dr. Julie Milstein of the Virology Division failed to find reverse transcriptase activity in these two lines, indicating that the cells were free of C-type retroviruses.

On September 4, 1980, Dr. Earley submitted starter cultures of both lines to the American Type Culture Collection. Vero 76 had been subcultured 22 times from the frozen CDC P128 cell pool. Therefore, it was submitted at P150. ATCC expanded this flask to P155 (PF27) to make their stock seed pool and designated this cell line as CRL 1587. Vero C1008 had been subcultured 11 times from the frozen CDC P4 cell pool. Therefore, it was actually P15 from the cloning procedure. ATCC expanded this flask, prepared their stock seed pool at P20 and designated this cell line as CRL 1586.

Further study of the different Vero cell lines was done between 1982 and 1984 when both of us worked at the United States Army Medical Research Institute for Infectious Diseases (USAMRIID) in Frederick, Maryland. The cloned C1008 cells were found to be superior for replication and plaque production of slow-growing non-cytolytic agents infecting man and rodents, notably the Hantaan group of Bunyaviruses. This experience confirmed and extended the observations made earlier at CDC.

A formal comparison was made between CCL 81 Vero and Vero 76 cells for quantitative assay of three distinct arenaviruses. This study was blinded for the virologist, Mr. Sherman Hasty, who performed the tests. The results of a 5 day double agar overlay protocol showed that for all three viruses Vero 76 cells yielded more (higher titer) and much sharper (clearly delineated) virus plaques, than CCL 81 cells.

Although we do not have a definitive explanation, nor any prognostic markers, it is now clear that different sublines of the original Vero cells have distinct biological properties when used to detect, replicate and assay different animal viruses. Because Vero cells are hypodiploid rather than diploid and because different maintenance environments have been employed over the years for cell growth it is not surprising that sublines with different properties have been unintentionally selected. It is noteworthy also that formal cloning experiments readily yielded lines which differ in morphology, metabolism, contact inhibition and viral "profile."

We believe that the most important lesson which emerges from this American lineage history is that Vero is *not* Vero is *not* Vero. Careful attention to pedigree may be required for optimum results with a given virus and it is suggested that intuitional manipulation and selection might produce further sublines of major utility. These observations serve mainly, however, to reinforce one inescapable conclusion: Vero cells have been one of the most powerful basic resources for the entire field of animal virology in the past quarter century. Vero will continue to be a major cell substrate for the virologist in years to come. We hope that this partial history of Vero cell lines in the Americas helps document its lineage in this 25th Anniversary year.

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3.

Biological Properties of VERO Cells

BIOLOGICAL PROPERTIES OF VERO CELLS DERIVED FROM THE PRESENT STOCK

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Morphology: A photograph, taken through Nomarski optics, of Vero (113 p) cells grown in a plastic dish is shown in Fig. 1. The monolayer showed moderate variation of cell size, and the majority of the cells contained 1 to 2 nucleoli per cell.

Growth Properties: Vero cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and antibiotics in an atmosphere of 5% CO_2-air. To obtain a single cell dispersion, the culture was treated with equal parts of 0.1% trypsin (Difco Labs.) solution and 0.04% EDTA solution. Plating efficiency, determined with exponentially growing cells, was 10 to 15%. The growth curve of Vero (115p) is shown in Fig. 2; this was determined with replicate cultures initiated with 10^5 plateau-phase cells per 60-mm plastic dish and subjected to medium renewals at appropriate interval after incubation for 5 days. The doubling time of the cells in the period of exponential growth was approximately 23 hours.

The cell cycle parameters as determined by frequencies of labelled mitoses, labelled cells and mitotic cells in actively growing population (3 days after seeding) were essentially consistent with those obtained from the cytofluorographic profile of cells stained with propidium iodide solution. Assuming exponential growth of cells in the culture, the fractions and the approximate duration of cells in the G_1, S, G_2 and M periods were shown in Table 1.

The saturation density determined from the plateau level of cell number was 6×10^5 cells/cm^2 reflecting a relatively tightly packed growth of cells. This density would imply that cells were located ca. 13 μ apart from each other if they were regularly arranged.

Anchorage independence was tested by seeding Vero cells into agar medium containing 0.3% Noble agar (Difco Labs.). When exponentially growing cells of Vero (118 p) were seeded into an agar layer at the concentration of 10^4 cells per dish, about 1% of cells formed colonies after incubation for 14 days. This indicates that a minor fraction of the population had become anchorage-independent by the 118th transfer generation.

Fig 1. Photograph of Vero cells at passage 113 taken through Nomarski optics.
(Courtesy of Professor Toshio Nagano, Department of Anatomy, School of Medicine Chiba University)

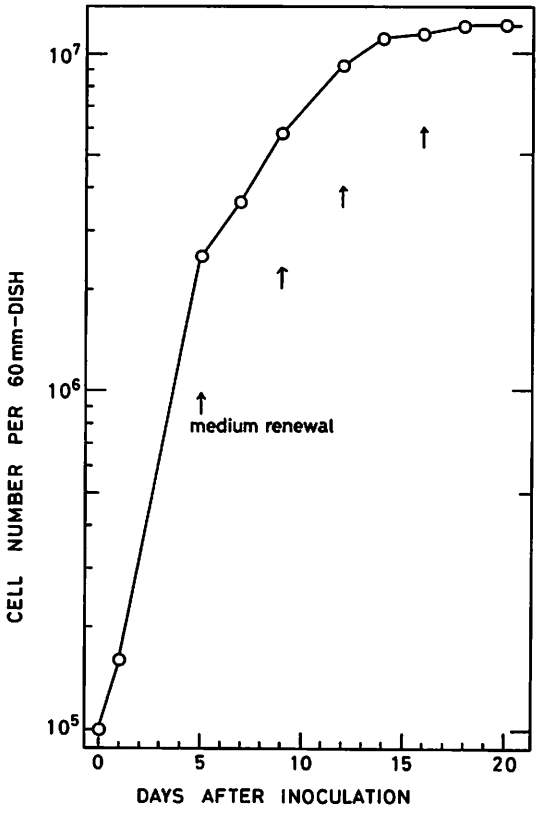

Fig. 2 Growth curve of Vero cells at passage 115.

Table 1. The Cell Cycle Parameters of Vero Cells

Period	Fraction (%)	Duration (hour)
G_1	62.9	10.8
S	32.0	10.5
G_2	3.7	1.2
M*	1.4	0.5
Total	100.0	23.0

*M represents cells ranging from metaphase to telophase.

Tumorigenicity: The tumorigenicity of Vero cells was examined by using Balb nu/nu mice which were produced in the breeding unit of NIRS. Vero (117 p) cell suspension was injected subcutaneously into 9 nude mice at 5 weeks of age. These mice had been irradiated with 400 rad of X-rays 1 day before inoculation. Three groups of nude mice, 3 mice each, were given 0.2 ml of a suspension containing 5×10^4, 5×10^5 or 5×10^6 Vero cells grown for 6 days. After 4 months of close observation for palpable growth, the mice were sacrificed. Careful dissection revealed that none of the mice showed any tumor development.

Sensitivity to radiations: Vero (120 p) cells were grown in MEM with 10% fetal calf serum. Cells were dispersed 4 days after seeding. Singly dispersed cells seeded into plastic dishes in appropriate numbers were irradiated with graded doses of 200 KeV X-rays and UV light, respectively. After incubation of the dishes for about 2 weeks, the cells were fixed and stained, and developed colonies were scored as survivors. Fig.3 shows a plot of surviving fraction of cells

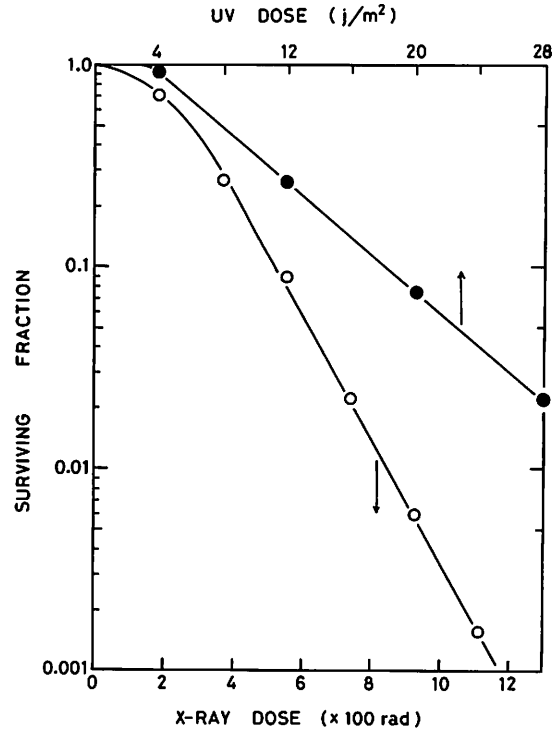

Fig. 3 Surviving fraction of Vero cells after exposure to various doses of X-rays (open circle) and UV light (closed circle).

against X-ray and UV light dose. The survival curve assumed a sigmoidal shape, as found for most mammalian cells. The X-ray survival curve started with a slight, negative slope, which increased as the dose increased, and finally became exponential after 400 rad. From the survival curve, we obtained values of 144 rad for D_0 and 3.7 for n (D_0, the mean lethal dose, is the dose required to reduce survival to 37% in the exponential region of the curve; n, the extrapolation number, is the intercept of the extrapolated exponential portion of the survival curve on the ordinate). The mean lethal dose of UV light was about 8.4 joules/m^2.

CYTOGENETIC EXAMINATION OF VERO CELLS DERIVED FROM THE PRESENT STOCK

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Vero cells at the 115th passage generation were grown in Eagle's MEM with 10% fetal calf serum and antibiotics for the present chromosome study.

Vero cells in the exponentially growing phase (3 days after initiation of culture) were treated with a drop of colcemid solution (GIBCO; 10 μg/ml) added to the culture medium for 2 hours at 37°C and removed carefully by the use of 0.1% trypsin (Difco Labs.) solution with minimum loss of loosely attached mitotic cells. Cells were then washed in PBS and finally suspended in 0.56% KCl solution for hypotonic treatment. After 25 minutes of hypotonic treatment in a water bath at 37°C, cells were fixed in 3-4 ml of acetic acid-alcohol (a mixture of 3 parts of ethanol and 1 part of glacial acetic acid) for about 1 hour. A drop of cell suspension at an appropriate concentration was spread on a clean slide glass in a fully humidified atmosphere and air-dried. Chromosomes were stained with 2% Giemsa solution and cells showing well spread metaphase chromosomes were photographed for enumeration of chromosome numbers and karyotypic analysis.

Fig. 1 shows a frequency distribution of chromosome numbers obtained from 156 metaphase plates. About 90% of total metaphase plates scored had chromosomes ranging from 52 to 62 and the majority of cells (86%) showed a chromosome number of 59 or less. The results indicate that Vero cells of the present stock have a subdiploid number of chromosomes, since the diploid chromosome number of African green monkey has been reported to be 60 (1). The peak was found at 58 and 70% of the cells were in the range of 56-60. The cells showing the normal diploid chromosome number ($2n = 60$) amounted to about 9% of the total cells observed. This was not consistent with the result in another subline of Vero cells, in which a pseudodiploid range of chromosomes was found even at 201 passages (2).

Fig. 2 and 3 showed a whole spread of metaphase chromosomes in a cell with 60 chromosomes and a classification of the constituent chromosomes based on their shape and size. It is clear that the karyotype is entirely different from that of normal diploid cells. As shown in Fig. 3, the chromosomes could be morphologically classified into three types, namely,

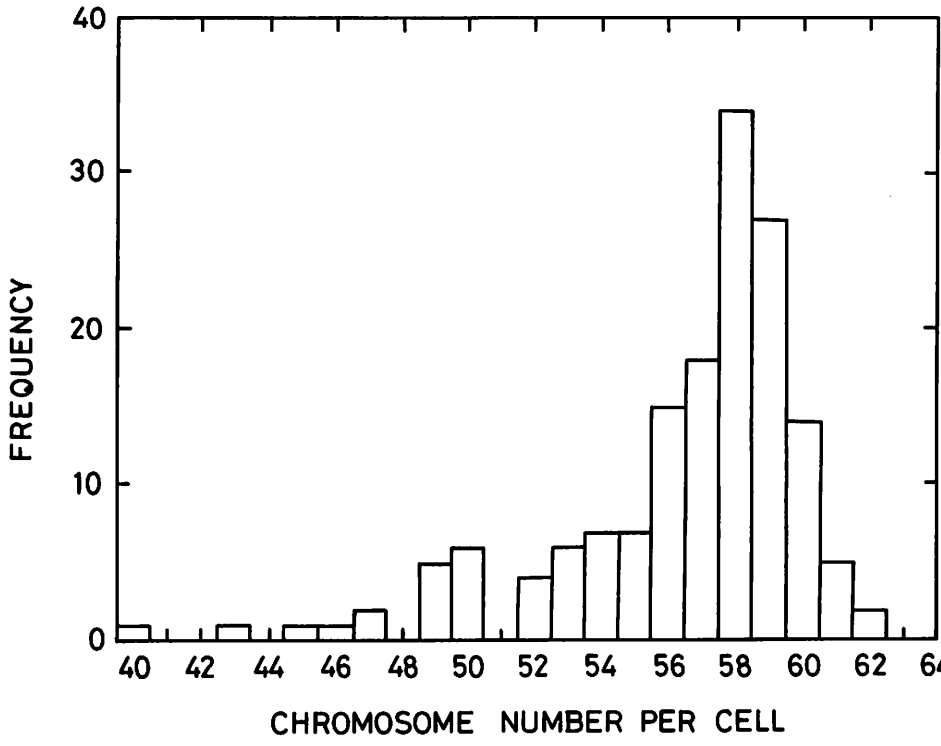

Fig. 1 Frequency distribution of chromosome numbers of Vero cells obtained from 156 metaphase plates.

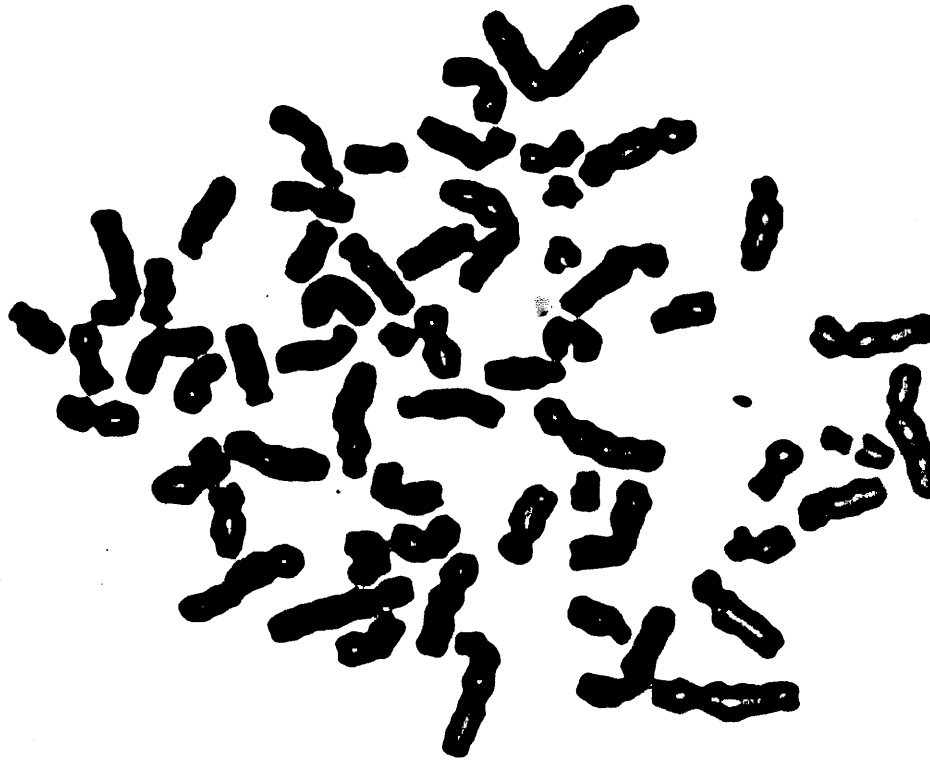

Fig. 2 Metaphase chromosome spread of a Vero cell with 60 chromosomes.

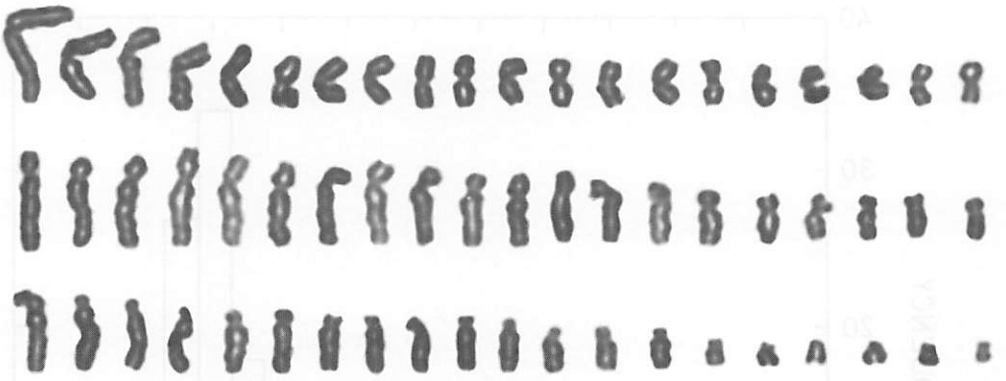

Fig. 3 Classification of the chromosomes shown in Fig. 2.

metacentric, submetacentric and subtelocentric types. Secondly, there was a significant variation in morphology among chromosome constituents. Several extraordinarily long-armed metacentric and submetacentric chromosomes were found without corresponding counterparts. In normal African green monkey cells, metacentric, submetacentric and subtelocentric constituents were found in 7, 12 and 10 chromosome pairs, respectively, in a total of 29 autosome pairs (1). It was reported that a few trisomies and monosomies were present in Vero cells of the other line (2). Identification of chromosome pairs in the present Vero cells, in relation to the normal diploid chromosomes of African green monkey cells, appeared to be nearly impossible. It was presumed that the structural changes of chromosomes found in our Vero cells had been induced over the period of 100 passages as a result of translocations or mutual exchanges of the whole or part of chromosome arms between the constituents.

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4.

VERO Cells in Virology

Introduction

Many viruses kill the cultured cell in which they multiply. As a result, the infected cell monolayers gradually develop visible evidence of cell damage, until eventually, all the cells within the culture container are damaged. These pathological alterations are called cytopathic effect (CPE). Most CPE can be observed by light microscopy without fixing and staining the cells. Thus in diagnostic virology, confirmation of CPE is an important first step. Accordingly, when isolating viruses from clinical specimens, the selection of cells which present clear CPE is desired.

If the infected cells are overlaid with agar medium to make certain that the spread of progeny is restricted to the vicinity of the originally infected cell, each virus gives rise to a localized focus of infected cells, and becomes large enough to see by the naked eye as an area of CPE (plaque) after a few days. Usually such monolayers are stained with a vital dye; the living cells take up the dye and the plaque appears as unstained clear areas. Counting the number of plaques is the most sensitive titration method for infectious viruses. In addition, cloning of viruses can be accomplished by this method. Thus, tissue culture is essential in basic research of viral diseases. Tissue cultures are also required in the production of antigens for serologic diagnosis and vaccine production. The main requirements for these processes are that the selected or specified viruses multiply well and the products remain free of contaminating agents. Since there are no cells which satisfy all the requirements for a variety of needs, it is essential to select the most appropriate cell lines for the virus studied.

Soon after isolation of the Vero cell line in 1963, the occurrence of CPE and the formation of plaque by a variety of viruses implemented an important research cell culture, not only in diagnostic virology but also in basic virology (p. 42). In 1966, it was found that the Japanese encephalitis virus caused CPE, including the formation of plaques on Vero cells (p. 51). Since then, Vero cells have become widely used in studies on arbo (arthropod-borne) viruses which include Japanese encephalitis virus. Therefore Vero cells are now used as the cell line of choice in the identification and isolation of unidentified arboviruses.

Vero cells were first brought to the National Institute of Health (NIH) of the United States in 1967. At that time, a research group was attempting to identify the etiologic viruses of Argentinian and Bolivian hemorrhagic fever in hope of being able to control these viral diseases. However, there were no susceptible cell cultures available for these hemorrhagic fever viruses. The introduction of Vero cells into their research lead to the discovery that the Tacaribe virus, the prototype of these hemorrhagic fever viruses, caused CPE. Thereafter, the pathogenic Junin (Argentinian hemorrhagic fever) and Machupo (Bolivian hemorrhagic fever) viruses were found also to cause CPE in Vero cells (p. 61). Then, as host cells, Vero cells were found to be useful for studies with *Arenaviridae* which contain the above-mentioned group of viruses. In addition the Vero cell became an essential tissue culture for the laboratory diagnosis of Lassa fever which is found in endemic areas of Africa (p. 66). This causative agent also belongs to a member of the *Arenaviridae* family.

There is another hemorrhagic fever virus group which belongs to *Bunyaviridae* and is found worldwide as etiologic agent of hemorrhagic fever with renal syndrome, HFRS. Since

tissue culture cells for these viruses were not established for sometime, the identification of these pathogenic viruses had not been possible. However, the virus which is responsible for Korean hemorrhagic fever was isolated and identified using mice. Soon after this discovery, it was found that this virus causes CPE on E6 cells, one of the mutant cell lines of Vero (p. 78). As a result, experiments on such dangerous viruses become possible in test tubes and the physicochemical properties of the viruses have been characterized well. Vero cells are found to be very useful in the isolation of other unclassified hemorrhagic fever viruses known as the etiologic agents of Marburg and Ebola hemorrhagic fever (p. 81).

For a long time, appropriate tissue cultures were not available for the multiplication of rubella virus; thus, characterization of this virus was difficult. However, recently, by the introduction of B-Vero cells, another variant Vero cell line, the biological and physicochemical properties of rubella virus have been characterized and analysis of the viral structural proteins have become possible (p. 84).

Shortly after the initial isolation of Vero cells, it was found that they were most suitable for the study of measles virus (p. 94). Although very rare, several years after being infected by measles, some patients were known to develop serious subacute sclerosing panencephalopathy (SSPE). When autopsy specimens of brain tissue from these patients were co-cultivated with Vero cells, a specific type of CPE developed, and the SSPE virus was isolated (p. 110). Thus, even today, in the study of measles and SSPE viruses, Vero cells are preferentially used. Also, studies of persistent infections by parainfluenza viruses are carried out using Vero cells for sometime (see Chapter 8).

The herpes simplex virus is known to cause a latent infection. Recently, antiviral agents have been developed for this virus and are being used clinically. For the study of their mechanism of action, latency and the characteristics of this virus, Vero cells have preferentially been used because of their clear CPE and high yield of virus (p. 123).

One of the unique characteristics of the Vero cell is that the cell does not produce interferon (p. 131). This is due to the fact that the cell does not contain interferon-producing genes (p. 138). Because of this Vero cells are useful for the study of basic characteristics of interferon. It is speculated that the reason Vero cells are susceptible to many viruses, particularly RNA type viruses, is that they do not produce interferon (see Chapter 8).

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Biological Characteristics and Viral Susceptibility of an African Green Monkey Kidney Cell Line (Vero)[1] (34285)

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A line of African green monkey kidney cells (Vero) was found useful for propagation and assay of the Tacaribe group of arboviruses, the etiological agents of South American hemorrhagic fever (1–3). Subsequently, Vero cells were shown to be useful in the assay of rubella virus (4–6), simian virus 5 (7) and certain adenoviruses (8, 9). Studies to determine the susceptibility of this cell line to additional viruses were further carried out. The present report provides an up-to-date list of the viruses so studied. Some of the biological characteristics of the Vero cells are also presented.

Materials and Methods. Viruses. The viruses tested in this study were obtained from various sources. They were passed once or twice in our laboratory under recommended conditions to obtain standard seed stocks and to produce various complement-fixing (CF) antigens for serological surveys (10).

Tissue cultures. Cell cultures other than Vero cells were obtained from Microbiological Associates, Inc., and were maintained at 37° on Eagle's minimum essential medium in Earle's balanced salt solution containing 3% agamma calf serum (obtained from Hyland Laboratories, Los Angeles, Calif.), 4 mmoles of glutamine, 100 U of penicillin, and 100 μg of streptomycin/ml. Vero cells in passage 93 were obtained from Dr. Y. Yasumura, Chiba, Japan, in 1964, and were maintained first at the Laboratory of Tropical Virology, National Institutes of Health, Bethesda, Md. (1–3), and later in this laboratory (4, 8). The Vero cells were subcultured and maintained in medium 199 supplemented with 5% fetal bovine serum (4). They were dispersed with 0.25% trypsin and diluted in the same medium. Cell suspension was adjusted to contain 1.5×10^5 cells/ml of medium. They were seeded into tubes (1 ml), 2-oz bottles (4 ml) or 32-oz bottles (40 ml) and were incubated for 3–4 days at 37°. The medium was then changed, and the cultures were incubated for an additional 2–3 days and were used for inoculation. The passage 107 through 165 levels of Vero cell cultures were used in this study. The passage 113 was frozen as stock, and was also deposited in the American Type Culture Collection repository.

Virus assay, assay for CPE of viruses. Two to four tubes were used for each 10-fold serial dilution tested. Observations for CPE were made for 28 days following inoculation, and fluids were changed every 5–7 days. The 50% end point was calculated by the Reed–Muench formula (11).

Plaque assay. Plaque assays in Vero cells were carried out by the double overlay method previously described (4). For the first agar overlay, the medium consisted of 0.5 % lactalbumin hydrolysate in Earle's salt solution, 2% fetal bovine serum, 0.23% $NaHCO_3$, antibiotics, and 1.5% agar. The second overlay was similar to the first, except that it contained neutral red at 1:45,000.

Neutralization tests. The constant virus-varying serum method, as well as the constant serum-varying virus method, were used for tube and plaque neutralization tests. Hyperimmune rabbit and monkey sera were mixed with the respective viruses, incubated at room temperature for 1 hr, and inoculated into Vero cell cultures.

[1] This work was supported by Contract PH 43-68-705 of the National Cancer Institute, National Institutes of Health.

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TABLE I. Chromosome Number of Vero Cells.

Passage level	Chromosome no.																							Total counted cells
	37	39	40	41	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	62	77	86	
122	3	1	1	1	2	5	2	3	4	2	7	15	7	12	12	4	10	1	4	2	1		1	100
141		1			3	3	2		2	2	6	5	9	12	28	15	8	2	1			1		100

Complement fixation. Preparation of CF antigens from infected Vero cultures was done according to the method described previously for viral and tumor antigens (10). The CF tests were carried out in the microtiter technique described by Sever (12). The sera employed for the CF tests were standard antiviral reagents of the CF laboratory of the Laboratory of Viral Diseases, NIAID, National Institutes of Health. Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement.

Chromosome cytology. For chromosome studies, Vero cultures were subcultured in the usual manner. After 6 hr, they were treated for 16 hr with diacetylmethyl colchicine (Coleceimide, Ciba) at a final concentration of 0.6 μg/ml; and this procedure was followed by treatment for 15 min with 0.9% sodium citrate. The cells were then removed from the surface of the bottle by scraping, and the cell suspension was processed according to the technique for air-dried preparations described for human peripheral blood (13).

Results and Discussion. Growth characteristics of Vero cultures. The Vero line assumed the characteristics associated with established cell lines. The cells grew in a dense, tightly packed sheet. Practically all of the cells were of polygonal morphology, some multinucleate. Fibroblastic cells were rarely observed (Fig. 1). Figure 1 shows that "transformation" as described by Chang (14) was not observed in the Vero line. The simian origin of Vero cells was confirmed by fluorescent antibody species determination (15). The Vero cells grew fast, in a dense, tightly packed sheet, and had higher plating efficiency than BS-C-1 cells, a continuous kidney cell line derived from the African green monkey (16). The BS-C-1 line grows very slowly in our hands.

Studies to determine the rate of multiplication of Vero cells were carried out with passage 129 cultures. Growth experiments initiated with approximately 1×10^5 cells/ml in 2-oz bottles indicated that the cells doubled in 72 hr and tripled in 4 days (Fig. 2).

In preparing large quantities of Vero cells for the experiments described in this report, it was found that the cell sheet from one C-2 bottle (area, approx 20 cm^2) yielded sufficient cells for preparation of 20 tube cultures, or one 32-oz bottle culture. Such cultures were ready for use after 5–7 days of incubation and one change of medium at about the third day. Throughout all the passage levels, the same procedure was employed.

Up to the present time, no pleuropneumonia-like organisms (PPLO) or latent viral agents have been found in Vero cultures maintained in this laboratory. No tumorgenicity of this cell line has been found in hamsters so far. On numerous occasions, cultures of the Vero line, as monolayers in fluid medium, have been shipped by air express to other parts of the world, and usually have survived the vicissitudes of travel with no difficulty.

Storage of the Vero cell line. Propagation of Vero cells from cultures which had been stored in the frozen state for varying periods of time, up to 12 months, was readily accomplished. Attempts were made to test the viability of Vero cells with 7.5% dimethylsulfoxide and medium 199 containing 10% fetal bovine serum in a nitrogen freezer. After thawing, viable-cell counts were performed, using 0.1% trypan blue as diluent. After 12 months of the nitrogen storage, 50–70% of virable cells were recovered, on 10 occasions, with an average of 55%. Cultures derived

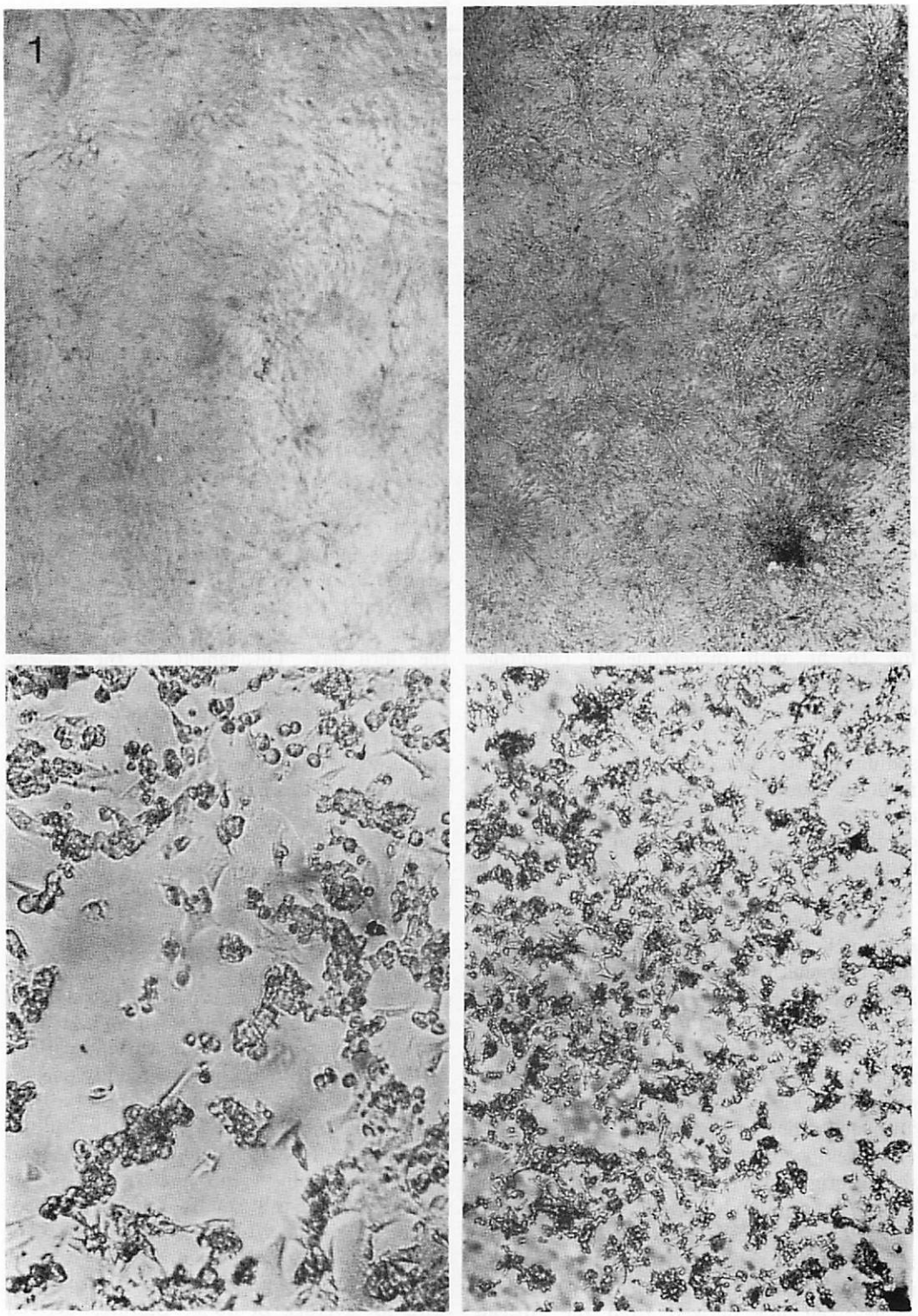
1

FIG. 1. Cytopathic effect (CPE) of adenovirus type 11 in Vero cells: (upper left), uninoculated Vero cells (×40); (upper right), CPE of adeno 11 at 37° (×40); (lower right), CPE of adeno 11 at 30° (×40); (lower left), CPE of adeno 11 at 30° (×115).

from the passage 122 and 141 levels, after storage for several months, were examined for SV-40 susceptibility. Both cultures grew well and the revived cells were equally as sensitive to SV-40.

Chromosome studies. Karyology of two different passage levels of the Vero cell line was studied. Continuous *in vitro* cultivation of the line proves to have changed the ploidy of the host species chromosome number. At passages 122 and 141, there was a heteroploid state. No unusual changes were observed in the examination of metaphase spreads: there were no distinctive markers, rings, or consistent breaks. Examination of passage 122 demonstrated that the model number of chromosomes fell in the region of 51–55, with emphasis on 52, 54, and 55. Passage 141 showed a region between 54 and 56 with the emphasis on 55.

A hundred metaphase spreads were counted

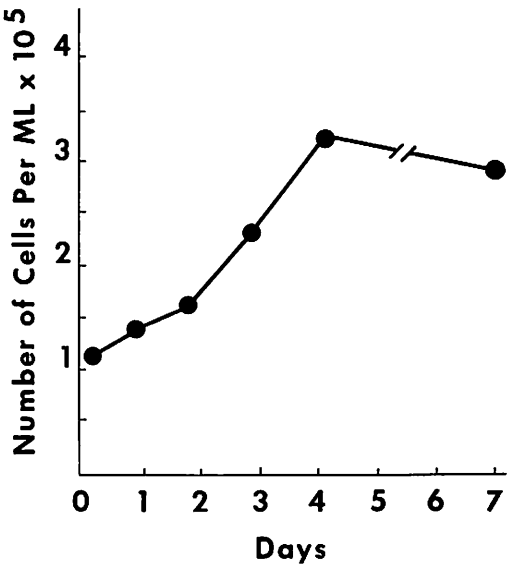

FIG. 2. Multiplication of Vero cells in passage 129 culture.

TABLE II. Susceptibility of Vero Cells to Adenoviruses.

Type	Strain	MOI[d]	CPE in Vero (days post-inoculation)	Infectivity titer[a] in Vero	Infectivity titer[a] in HEK	CF titer[b] in Vero
Human adenovirus						
1		—	++ (4)	6.7	—	>128
2		—	+ (4)	7.2	9.7	>128
3	G.B.	300	+ (7)	6.7(6.0)[c]	9.2	64
4	38558	10	++ (4)	3.5	—	32
5		10	++ (4)	7.7	—	>128
6		10	++ (4)	7.2	—	>128
7	Gomen	300	++ (4)	5.2	8.2	>8
8		—	+++ (5)	6.7	—	—
9		—	+ (9)	3.2	—	—
11	Slobitski	10	++ (4)	5.2	9.2	32
12	Huie	32	++ (2)	6.7	8.2	>128
14	DeWit	100	+ (9)	4.7	7.2	64
16	CH 79	10	+ (5)	6.2	9.2	64
18	DC	3.2	+ (3)	5.2	6.2	64
21	D 56	30	+ (3)	7.7	8.2	>8
31		10	+ (3)	6.2	7.7	>128

[a] Infectivity titer expressed as $\log_{10}TCD_{50}$/ml.

[b] CF titer = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement.

[c] PFU/ml.

[d] MOI = multiplicity of infection; HEK = human embryonic kidney cells; and — = not done.

TABLE II. (continued) Part II.[a]

	Virus	MOI[d]	CPE[e] in Vero (days post-inoculation)	Infectivity titer[b] in Vero		In Vero	
				Vero	AGMK	PFU	CF[c]
	Simian adeno						
I	SV-36	—	++ (4)	6.2	—	—	—
II	SV-15 (m-4)	100	++++ (7)	7.2	8.2	—	—(80)[f]
	SV-17 (m-6)	1	++ (4)	6.2	6.7	—	16
	SV-23 (m-2)	10	++ (3)	6.7	7.7	—	—
	SV-32 (m-3)	300	++ (3)	8.2	8.7	—	—
	SV-37	100	+ (3)	7.2	9.2	—	—
	SV-39	—	++ (3)	7.7	9.2	7.2	16
III	SV-1 (m-1)	1/10	++ (3)	7.2	5.2	—	>128
	SV-20 (m-12)	10	++ (3)	7.7	7.2	7.6	>128
	SV-25 (m-8)	1/10	++ (4)	5.7	5.2	—	>128
	SV-30	1/10	++ (3)	7.2	5.2	—	>128
	SV-33 (m-10)	10	+++ (3)	8.2	5.2	8.3	>128
	SV-34 (A7644)	1/10	++ (5)	5.2	6.2	—	—
	SV-38	100	+ (3)	8.2	7.2	—	>128
	SA-7	10	+++ (3)	8.2	9.2	—	>128

[a] Each virus sced pool was titrated simultaneously in Vero cells and in the indicated control system; 2–4 tubes/dilution were examined daily for 28 days for CPE (cytopathic effects).

[b] Infectivity titer expressed as $\log_{10}TCD_{50}$/ml.

[c] CF titer = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement.

[d] MOI = multiplicity of infection; and — = not done.

[e] CPE: + = 11–25%; ++ = 26–50%; +++ = 51–75%; ++++ = >75% of cells affected.

[f] Hemagglutination titer.

in each passage. The results indicate that a heteroploid state now exists, with the model number of chromosomes falling in the range of 52–55 (Table I).

Infection of Vero cells with adenoviruses. It has been shown that the Vero cell line can be used for the production of adenovirus tumor (T) antigen relatively free from viral antigen by the thermal separation method (8), as well as for the production of high-titered viral antigen (8, 9). Further studies on the susceptibility of Vero cells to adenoviruses have yielded similar results. High-titered CF antigens for human and simian adenovirus were produced in Vero cells. Infectivity titers of simian adenovirus obtained in Vero cells were comparable to those obtained in primary African green monkey kidney cells. However, infectivity titers of human adenovirus obtained in Vero cells were 1–4 logs lower than those obtained in human embryonic kidney cells (Table II). Thus, the Vero cell line, which is relatively sensitive to human and simian adenoviruses, is more economical to produce than primary human

TABLE III. Cytopathic Effect (CPE) and Infectivity Titers of Adenovirus Type 11 in Vero Cell Cultures at 37 and 30°.

Temp of incubation (°)	Time of harvest (days post-inoculation)	CPE[a]	Infectivity titer[b] in HEK cells
37	4	+	5.4
	11	++	—
	15	++++	6.7
30	4	++	6.0
	11	++++	—
	15	++++C	7.2

[a] CPE: + = 11–25%; ++ = 26–50%; +++ = 51–75%; ++++ = >75% of cells affected; C = complete lysis of cells; and — = not done.

[b] Infectivity titer expressed as $\log_{10}TCD_{50}$/ml; multiplicity of infection was 100 TCD_{50}/cell.

and simian cells, and can be used as a substitute for them in the assay of these viruses.

It has also been found (8) that the highest titers of T antigen for certain adenoviruses were obtained in Vero cells incubated at 30°. Development of CPE and infectivity titers of adenovirus type 11 in Vero cell cultures incubated at 30 and 37° was examined. Vero cultures were inoculated with approximately 100 TCD_{50} per cell of adenovirus type 11 and incubated at 30 and 37° in a stationary position. The cultures were examined for CPE, and fluids were harvested and tested for infectivity at various intervals after inoculation.

There was not only a striking enhancement of CPE in the cultures incubated at 30°, but significantly more virus was obtained. (Fig. 1 and Table III). It is also true, in the case of a Japanese B encephalitis virus study (17) that there was a striking enhancement of CPE in the cultures incubated at 30°, as well as more virus (0.5–1 log) being produced.

Susceptibility to infection with other viruses. Vero cells were further examined for their susceptibility to various other groups of viruses, including herpes, myxovirus, papova, picorna, pox, reo, arbo, and rubella. This work has led to a satisfactory cytopathic and plaque assay for arboviruses, rubella, human and simian adenoviruses (18), SV-40 (19), polio, reoviruses, myxoviruses [measles (20), SV-5], herpes virus, and pox viruses (vaccinia, myxoma). Furthermore, complement-fixing and hemagglutinating antigen yields were also high, although only a limited number of viruses were tested (Table IV).

Since Vero cells were successfully used for

TABLE IV. Susceptibility of Vero Cells to Various Viruses Other than Adenoviruses.[a]

Group and virus (strain)	MOI[d]	CPE in Vero (days post-inoculation)	Infectivity titer[b] in Vero	Infectivity titer[b] in Indicator[c]	In Vero PFU	In Vero CF[e]
Herpes						
Herpes simplex	1/1000	++++ (3)	6.2	—	—	—
Varicella	—	++++ (7)	6.2	—	5.7	—
Human salivary gland	1/1000	++++ (7)	6.0	—	5.1	—
Canine herpes	1/1000	++++ (9)	6.2	—	—	—
Myxovirus						
SV-5	1/50	++++ (9)	6.2	5.7(RhMK)	5.5	1:32(128)[f]
Mumps	1/100	++++ (7)	5.2	5.2(AGMK)	5.7	—
New Castle	—	+++ (7)	6.7	—	6.3	—
Sendai	—	0 (28)	—	—	—	—
Respiratory syncitial	1/10	++++ (12)	6.2	6.2(AGMK)	6.7	—
Measles	1/50	++++ (5)	6.7	6.7(AGMK)	6.6	—
Bovine parafiu T.3[g]	—	++++ (5)	5.5	5.0(Bk)	—	—
Papova						
Polyoma	1/10	0 (28)	<1.7	6.7(MsK)	—	—
SV-40	10	++ (3)	8.2	8.2(AGMK)	8.2	>128
Picorna						
Polio Type 1	100	++++ (2)	8.7	8.2(AGMK)	8.9	—
2	100	++++ (2)	7.7	8.0(AGMK)	—	—
3	100	++++ (2)	8.2	8.2(AGMK)	—	—
Echo 11 (Gregory)	1/10	++ (3)	5.2	—	—	—
Coxsackie A-9	1/10	+ (5)	6.2	—	—	—
A-21	1/10	+ (10)	2.7	—	—	—
Pox						
Vaccinia	10	++++ (5)	7.2	7.2(AGMK)	7.4	—
Myxoma	—	++++ (6)	6.2	—	6.0	—
Fibroma	—	++++ (5)	5.2	5.2(MA-111)	5.0	—

TABLE IV (continued)

Group and virus (strain)	MOI[d]	CPE in Vero (days post-inoculation)	Infectivity titer[b] in Vero	Infectivity titer[b] in Indicator[e]	In Vero PFU	In Vero CF[c]
Reo						
Reo T. I (Lane)	10	++++ (8)	8.0	8.2(RhMK)	7.8	1:4
(716)	10	++++ (5)	7.7	8.0(RhMK)	7.8	1:32
II (4/48)	1/10	++++ (8)	6.2	—	—	—
III (Dearing)	10	++++ (5)	8.0	8.5(RhMK)	8.2	—
Arbo						
Sindbis	—	+++ (4)	5.7	—	5.8	—
Japanese B. encephalitis (Nakayama)	1/10	++++ (4)	9.0	8.5(HK)	9.4	—
Tacaribe (TR 11573)	1/1000	++++ (5)	8.2	8.1(SM)	8.4	—
Junin (XJ)	1/100	++++ (7)	7.8	7.7(SM)	7.7	—
Machupo (Machupo)	1/1000	0 (28)	—	—	8.8(7.5)[h]	
Amapari (BE An-70563)	1/100	+++ (5)	6.5	7.7(SM)	7.8	—
Vesicular stomatitis	1/10	++++ (3)	6.2	6.2(AGMK)	6.3	—
Miscellaneous						
Rubella (RV)	1/10	++++ (7)	6.4	6.0(AGMK)	5.7	1:8
(M-33)	1/10	++++ (8)	6.2	5.8(AGMK)	—	—
(HP-77)	1/10	++ (8)	3.5	3.7(AGMK)	—	—

[a] Each virus seed pool was titrated simultaneously in Vero cells and in the indicated control system. Two to four tubes per dilution were employed. Cultures were examined daily for 28 days for cytopathic effects (CPE).

[b] Infectivity titer expressed as $\log_{10}TCD_{50}$/ml.

[c] CF titer = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement.

[d] MOI = multiplicity of infection; — = not done.

[e] RhMK = rhesus monkey kidney; AGMK = African green monkey kidney; MsK = mouse embryonic kidney; BK = bovine kidney; MA-111 = a continuous newborn rabbit kidney cell line; HK = hamster kidney; and SM = suckling mouse.

[f] Hemagglutination titer.

[g] Bovine parainfluenza Type 3.

[h] Plaque forming units in MA-111.

the first time in the U.S.A. for assay of the Tacaribe group of arboviruses (1–3), a number of investigators have reported the susceptibility of Vero cells to infection with various viruses (Table V). Perhaps the most extensive use of Vero cells has been reported for the arbovirus group (1–3, 21–23). Each of 19 arboviruses from Central or South America produced plaques in the Vero cell line and in MA-111, a continuous cell line of newborn rabbit kidney. It has been suggested that Vero cells were superior to the MA-111 cell line (21). This was also true in the case of the Tacaribe group of arboviruses, as shown in comparison studies between the Vero and MA-111 cell lines (1–3).

It has also been shown that Vero cells were useful for primary isolation, propagation, and assay of rubella virus, in lieu of the widely used primary African green monkey kidney cells (4, 5), and this has been confirmed (6). In addition, a recent report showed that Vero cells would furnish a useful alternative for BHK-21 cells for production of rubella hemagglutinating antigen (25).

Summary. A continuous cell line derived from kidney tissue of the African green monkey (*Cercopithecus aethiops*), and designated as Vero, has been maintained and was studied at passage 107–165 levels for susceptibility to various viruses. Vero cells supported the growth of a number of viruses to high

TABLE V. Reported Data on Viral Susceptibility of Vero Cells.

Virus group	Virus	Ref.
Adeno	Adeno	Rhim *et al.* (1968) (8) Schell *et al.* (1968) (9)
Simian Adeno	SV-30	Slifkin *et al.* (1968) (18)
Papova	SV-40	Yasumura and Kawakita (1963) (19)
Myxo	Measles	Sasaki *et al.* (1964) (20)
	SV-5	Rhim and Schell (1967) (7)
Arbovirus		Earley *et al.* (1967) (21) Stim and Henderson (1968) (22)
Group A	EEE, VEE, UNA, Pixuna, Macambo and others	
Group B	Ilheus, yellow fever, SLE, Bussuquara	
Bunyamwera, Guaroa, Cache Valley, Wycomya California, B-878, B-1113-14 Changuinola, BT-104 Sandfly fever, Chagres vesicular stomatitis		
Tacaribe		
	Tacaribe	Simizu *et al.* (1967) (1)
	Junin	Rhim *et al.* (1967) (2)
	Machupo	Rhim *et al.* (1969) (3)
	Amapari	Pinheiro *et al.* (1966) (23)
	Rubella	Rhim and Schell (1967) (4) Liebhaber *et al.* (1967) (5) Rafajko and Zur Nedden (1968) (25) Desmyter *et al.* (1968) (6)
	African horse-sickness virus	Ozawa (1967) (24)

titers. Extensive cytopathic effects and plaque formation in Vero cell cultures make possible an efficient and reproducible direct technique for the demonstration of a number of viruses and of specific neutralizing antibodies. In addition, growth of a number of viruses (such as rubella, SV-5, and the Tacaribe group of viruses) to a high titer in a continuous simian cell line provides a convenient source of virus and antigen for other studies. Thus, the Vero cell line provides the virologist with another tool for diagnostic and research work, and perhaps offers advantages for large scale production of viral agents for vaccine.

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COMPARATIVE STUDY ON NEUTRALIZATION TESTS OF JAPANESE ENCEPHALITIS

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The serological studies of Japanese encephalitis (JE), especially its serological diagnosis and sero-epidemiology, have been developed owing to hemagglutination of JE virus discovered by Sabin[1] and hemagglutination inhibition (HI) test established by Clarke and Casals[2].

The titers of HI antibodies in human and animal sera are measured easily and accurately by Clarke's method. And much more facts on serological surveys of JE have been proved than by complement fixation test.

On the other hand, recent advances of tissue culture method make it possible to determine neutralizing antibody simply and uniformly *in vitro*. By those methods, the diagnoses and epidemiology of poliomyelitis, coxsackie, etc. have been markedly developed.

JE virus grows well in various kinds of animal tissue cell. However, it had not shown any cytopathic effects (CPE) nor plaque formation in many tissue culture systems.

Recently Kissling[3], and Hammon and his co-workers[4] found CEP of JE virus in tissue cultured hamster kidney cell. Porterfield[5] found plaque formation with JE virus in chick embryonic fibroblast monolayer culture.

These discoveries have promoted virological studies of JE in tissue culture and also have harvested many valuable results of epidemiological study on this disease.

In our previous study[6], it was recognized that neutralization test of JE could be performed by plaque reduction technique in bottle cultured chick embryonic fibroblast and neutralizing antibody titers of human sera were significantly correlated with HI antibody titers.

In this series of experiment, we attempted to do neutralization tests by employing a stable cell line of green monkey kidney, designated as Vero cell which was sensitive to JE virus.

Our neutralization tests with Vero cells were done by plaque reduction method in cultured bottle and cytopathic effect method in cultured tube. Furthermore, plaque inhibition zone test developed by Porterfield[7] and DeSomer[8] was applied to the neutralization test of JE with bottle cultured chick embryonic fibroblast.

A part of this study was presented at the 12th General Meeting of the Society of Japanese Virologists, Tokyo, November, 1964.

Materials and Methods

1. Human sera: Seventy human sera of infants and adults were kindly supplied by Dr. Sunaga, Nagoya University, Nagoya and Dr. Ashidate, Tokyo Municipal Toshima Hospital, Tokyo.

2. Virus: Nakayama strain virus was used throughout all experiments. Ten percent infected mouse brain suspension with Hank's solution contained 0.55% bovine albumin was centrifuged at 10,000 rpm for 30 minutes. The supernatant was distributed in glass capillaries and stored at −70C until use. These stocks of the virus were preliminarily tested for determining plaque forming units (PFU) and TCD_{50} titer for Vero cells and also for determining plaque forming units for chick embryonic fibroblast. In the experiments, 60–80 PFU virus/0.1 ml and 30–50 TCD_{50} virus/0.1 ml were used for Vero cells cultured in bottle and tube, respectively.

Hank's solution contained 0.55% bovine albumin was used as the diluent for human sera and virus suspensions in neutralization tests.

3. Vero cells: A stable cell line of green monkey kidney, Vero cell,[9] was established by Dr. Yasumura, Chiba University, Chiba and supplied from him. Growth medium for Vero cells consisted of 2% calf serum (JE viral antibody free), 0.5% lactalbumin hydrolysate, 0.1% yeastolate, 0.1% polyvinylpyroridon and 97.3% Earle's solution. For a maintenance medium for tube culture, Earle's solution contained 0.5% lactalbumin hydrolysate was used.

Tubes or 2-oz bottles were seeded with 1 or 5 ml of a suspension contained 1.5×10^5 cells per ml, respectively. These cultures were incubated at 37C and complete cell monolayers were obtained on 5–6 days after seeding. Then tubes were replaced with maintenance medium and soon used for test. Bottles were renourished with fresh growth medium for a few more days and then used for test.

The constituent of agar overlay for cultured bottles was as follows:

A. 3% Agar (Noble, Difco)			90.0 ml
B. 10×Earle's solution (without phenol red and $NaHCO_3$)			18.0 ml
5% Lactalbumin hydrolysate			9.0 ml
5% Yeastolate			3.6 ml
7.5% $NaHCO_3$			5.4 ml
0.1% Neutral red			3.9 ml
Antibiotics	Penicillin	200,000 units/ml	0.5 ml
	Streptomycin	200,000 μg/ml	
Water			49.6 ml

Six ml of the mixture of the above two solutions, A and B, was poured into a cultured bottle.

4. Chick embryonic fibroblast monolayer: Eleven days' eggs were used. Square bottles (70 ml) were seeded with 5 ml of a cell suspension containing 4×10^6 cells per ml. After 18–20 hours' incubation at 37C, bottles were used for experi-

ments. Growth medium for chick embryonic fibroblast consisted of 0.5% lactalbumin hydrolysate, 10% calf serum (JE viral antibody free) and 89.5% Hanks' solution.

The constituent of agar overlay was as follows:

A. 3% Agar (Noble, Difco)	90.0 ml
B. 10× Earle's solution (without phenol red and $NaHCO_3$)	18.0 ml
5% Lactalbumin hydrolysate	9.0 ml
7.5% $NaHCO_3$	5.4 ml
0.1% Neutral red	5.0 ml
Antibiotics	0.5 ml
Water	52.1 ml

Five ml of the mixture of the above two solutions, A and B, were poured into a virus-infected square bottle.

5. *Procedure of neutralization tests:* Aliquots of serially 4-fold diluted serum were mixed with equal volume of selectively diluted virus suspension. The virus-serum mixtures were incubated at 37C for 60 min. Two tenths ml of the mixture was inoculated into Vero cells cultured bottle or tube. Four bottles for plaque reduction method and three tubes for cytopathic effect (CPE) method were used for each virus-serum mixture, respectively.

For unneutralized virus adsorption, inoculated bottles were placed at 37C for 90 min. and then overlaid. Bottles were turned over and incubated at 37C. Number of plaques was counted on 10 days after inoculation.

Tubes inoculated with the above virus-serum mixture were also placed at 37C and CPE was examined for 10 days after inoculation.

For the virus control, aliquot of selectively diluted virus suspension was mixed with equal volume of Hanks' solution contained 0.55% bovine albumin and 0.2 ml of the mixture were inoculated into bottles or tubes after the incubation at 37C for 60 min.

6. *Plaque inhibition zone test*: Two tenths ml of virus suspension contained 1000–2000 PFU viruses, was inoculated onto chick embryonic fibroblast monolayer in square bottles. These bottles were incubated at 37C for 90 min. for virus adsorption and overlaid with 5 ml of agar overlay. After the agar solidified, filter paper disks (6 mm in diameter) which contained the serum to be tested, were placed on the surface of the agar. Bottles were turned over and incubated at 37C. The diameter of plaque inhibition zone was measured on 6 days after inoculation.

7. *Hemagglutination inhibition (HI) test*: The procedure of this test was conformed to the method described by Clarke and Casals. Sera were pretreated with kaolin and goose red cells. Serial 2-fold dilutions of sera were tested against 8 units of HA antigen prepared with Nakayama strain virus.

Results

1. Plaque reduction test

Mean number of plaques in four bottles which were inoculated with each diluted serum-virus mixture was calculated. Serum-dilution titers of antibody were calculated by extrapolation to find the dilution of serum which produced 50 percent fewer plaques than those presented in virus control bottles. And its serum-dilution titer of antibody was arranged for neutralizing antibody (NT) titer of the serum.

HI and NT titers of 59 human sera were obtained in each test. Those titers are plotted in Fig. 1.

The result is quite similar to that obtained in our previous study with chick embryonic fibroblast culture. Namely there is a linear relationship between the

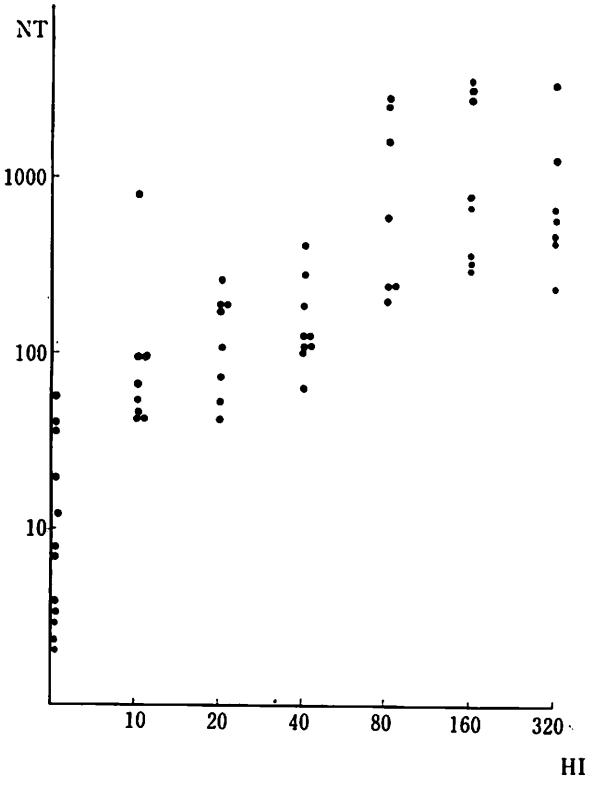

Fig. 1. *Relationship between HI and NT titers obtained with plaque reduction method.*

NT and HI titers of serum samples. In addition, by using this method, neutralizing antibody was detected in over half number of sera without HI antibody.

2. Neutralization test by cytopathic effect (CPE) method

In this method, NT titer of the serum tested, was calculated for the 50 percent neutralizing end point per 0.1 ml against the challenge virus dose (30–50 TCD_{50}/0.1 ml) by the method of Reed and Muench.

NT titers in this test and HI titers of human sera are plotted in Fig. 2.

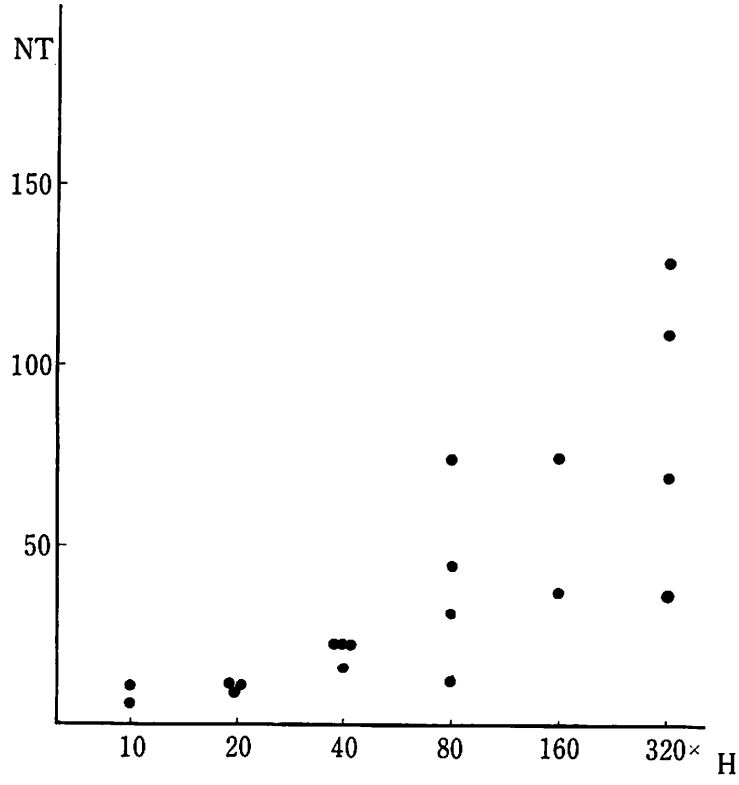

Fig. 2. *Relationship between HI and NT titers obtained with cytopathic effect method.*

As shown in that figure, only limited sera with HI titer higher than 40× showed some detectable neutralizing antibody, and NT titers in this test were found to be lower than the titers obtained in HI test.

3. Neutralization test with plaque inhibition zone method

Forty-eight human sera were tested by this method.

The relationship between HI titer and the area of plaque inhibition zone which

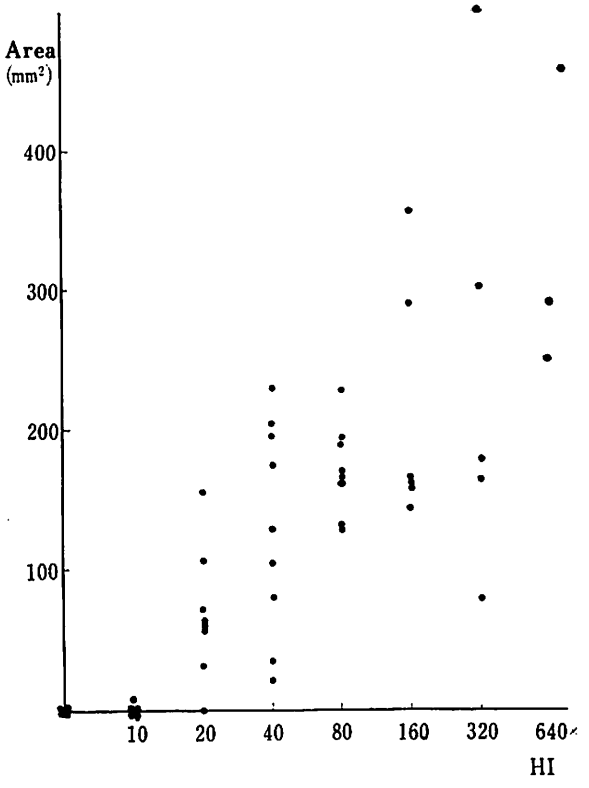

Fig. 3. *Relationship between HI titer and the protected area obtained in plaque inhibition zone test.*

was produced by filter paper disk contained the human serum, is shown in Fig. 3. And it seems there are a close correlation between them. In this test, only limited sera with HI titer at 20× or more showed detectable plaque inhibition zones.

Discussion

Recently, HI test took place of complement fixation test which is employed as a serological test of JE.

However, the determination of NT titer in human and animal sera is frequently required for epidemiological survey of JE. Usually, the method of neutralization test of JE is performed by decreasing virus-constant serum method with mice. But this test has not been used so widely as it has been restricted by consuming time and economy. And also this test is done by decreasing virus-constant serum method, the result obtained is not directly compared with the

result obtained by HI test which is conducted by constant virus antigen-decreasing serum method. In our previous study, we performed neutralization test by constant virus-decreasing serum method using plaque reduction technique in chick embryonic fibroblast culture. And we recognized that the results obtained in this test was significantly correlated with the results in HI test.

In this study, we carried out three kinds of neutralization test also by constant virus-decreasing serum method in new tissue culture system with individual human serum and we compared the above results with the results in HI test. Three kinds of neutralization test performed were plaque reduction neutralization test with Vero cells, tube neutralization test (CEP method) with Vero cells and plaque inhibition zone test with chick embryonic fibroblast monolayer.

Now, we attempt to find comparative advantages among above three tests and plaque reduction test with chick embryonic fibroblast which was already reported.

Plaque reduction neutralization method using either Vero cells or chick embryonic fibroblast, is extremely more sensitive than HI test. Because the NT titers obtained are usually higher than the HI-titers and over half number of sera without HI antibody had some detectable neutralizing antibody. But one cannot test so many samples at one time by this method as by HI test. And this method is needed a few more days than HI test.

The procedure of tube neutralization test (CEP method) with Vero cells is very simple. However, this method is less sensitive than HI test and needs limited virus dose for challenge virus. Unless one uses limited virus does (30–50 TCD_{50}/0.1 ml) for challenge virus, the antibody titer obtained is not reproducible frequently.

Plaque inhibition zone test is performed with very small volume (about 0.005 ml/disk) of sample and many samples are able to be easily tested at one time. However, this method is less sensitive than HI test.

In consideration of taking advantage or disadvantage of these methods, one must select a suitable method e. g. an accurate method or a simple method or a rapid method for his own purpose.

Summary

The neutralization tests of Japanese encephalitis were performed by plaque reduction and cytopathic effect methods using a stable cell line of green monkey kidney (Vero cell).

Plaque reduction method with Vero cells was much more sensitive than cytopathic effect method. And it was as sensitive as plaque reduction method which was already studied with chick embryonic fibroblast.

Plaque inhibition zone test with filter paper disk on chick embryonic fibroblast monolayer, was found to be also a useful method as a neutralization test of Japanese encephalitis.

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Explanation of Plates

Plate—1. Cytopathic effect induced by Japanese encephalitis virus (Nakayama strain) in Vero cells. 300×
Fig. A. Uninoculated control.
Fig. B. Slight effect.
Fig. C. Severe effect.

Plate—2. Plaque inhibition zone in chick embryonic fibroblast monolayers, produced by human serum contained in filter paper disk (black circle).
Each paper disk contained the serum with the HI titer mentioned on bottle, respectively.

Plate-1

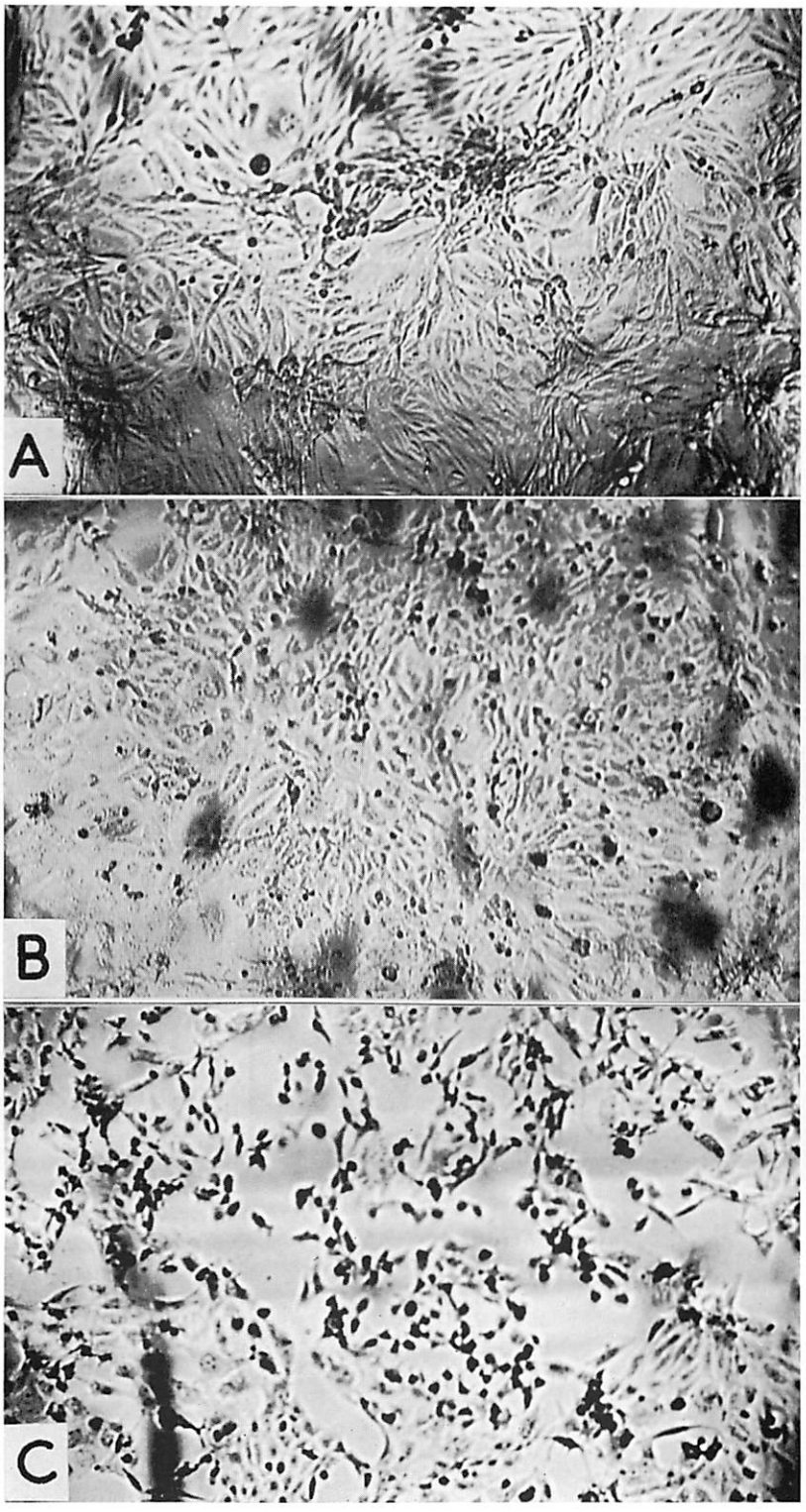
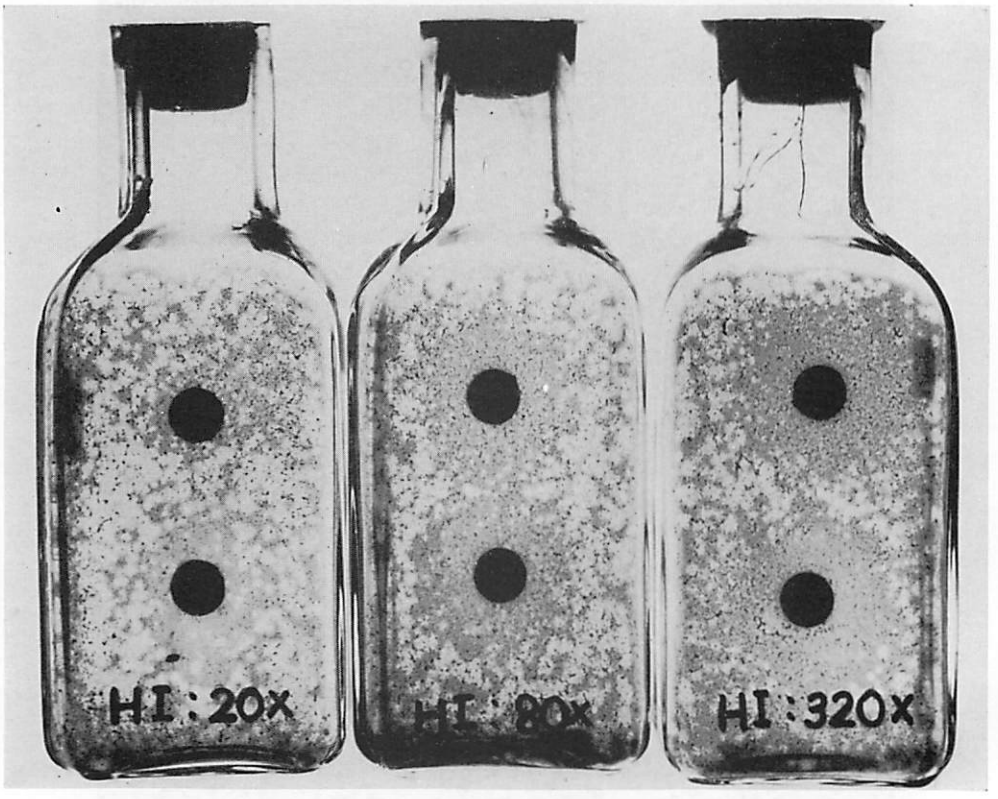


Plate-2



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Characterization of the Tacaribe Group of Arboviruses. 1. Propagation and Plaque Assay of Tacaribe Virus in a Line of African Green Monkey Kidney Cells (Vero). (32029)

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The propagation of Tacaribe virus in HeLa cells(1) and in hamster kidney tissue culture (HKTC)(2) has been described. However, marked and consistent cytopathic effect (CPE) following inoculation of Tacaribe virus has not been observed. Only incomplete CPE was noted in HeLa cells and a slight CPE in HKTC. Tacaribe virus has been shown to produce plaques under agar overlay without CPE in primary rhesus monkey kidney(MK)(3), continuous embryonic rhesus MK (MA-104)(4), and continuous newborn rabbit kidney (MA-111)(5) cell cultures.

This report describes CPE and plaque formation with Tacaribe virus in monolayer cultures of an African green MK *(Cercopithecus aethiops)* cell line (Vero). It was found that Vero cells not only support Tacaribe virus growth, but also develop cytopathic change with complete destruction of the cell sheet. The Vero cell cultures thus furnish a useful TC system for assay and propagation of Tacaribe virus.

Materials and methods. The virus used in this study was the TR 11573 strain of Tacaribe virus supplied by Dr. W. Downs. It had been through 21 passages in suckling mouse(SM) and one in suckling hamster (SH). Antiserum for Tacaribe, Junin and Machupo viruses was obtained from rabbits inoculated intravenously 3 or 4 times at 7-10 day intervals with a suspension of infected SM or SH brain diluted to 10^{-8} in Medium 199 (M-199)(6). Vero cells(7) were kindly furnished by Dr. Y. Yasumura, Chiba University, Chiba, Japan, in 1964. In this laboratory, the cells have been subcultured in M-199 containing 5% fetal calf serum, and maintained in M-199 with 2% fetal calf serum. They were dispersed with 0.25% trypsin and diluted in the growth medium. Cell suspension was adjusted to contain 1.5×10^5 cells per milliliter of medium. They were seeded into tubes (1 ml), 2 oz. bottles (5 ml), or 32 oz. bottles (40 ml), and were incubated for 3 to 4 days at 37°C. The medium was then changed, and the cultures were incubated for an additional 2 to 3 days and were ready for inoculation. Medium 199 without serum was used as virus and serum diluent. For the agar overlay, the medium was made by mixing equal parts of 3% agar in sterile distilled water and the following:

Lactalbumin hydrolysate (5%)	12.0 ml
Earle's balanced salt solution (without $NaHCO_3$) 10x	18.0 ml
$NaHCO_3$ (7.5%)	5.4 ml
Fetal calf serum (inactivated at 56° for 30 minutes)	3.6 ml
Neutral red (0.1%)	4.0 ml
Antibiotics (200 U penicillin, 200 γg streptomycin)	1.0 ml
Sterile distilled water	46.0 ml

Virus assays. Cell cultures in tubes were drained and inoculated with 0.2 ml of test material. Four tubes were usually used for each 10-fold serial dilution tested. After 1 hour of incubation at 37°C, 1 ml of medium was added. The tubes were incubated in stationary racks at 37°C, and examined for development of CPE. Final readings were made on the 12th day following inoculation and the 50% infectious endpoint was calculated by the Reed-Muench formula(8).

For *plaque assay,* monolayers in 2 oz. bottles were inoculated, in duplicate, with 0.2 ml of appropriate virus dilutions after removal of the medium and washing once with diluent.

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TABLE I. Serial Passage and Comparative Virus Titers of Tacaribe Virus (TR 11573 Strain) in Vero Cells and Suckling Mice.

Passage level	Cumulative $\log_{10}$ dilution of original inoculum*	Cumulative No. of days in tissue culture	Log_{10} virus titer		
			TCD_{50}	PFU	SM† LD_{50}
SM/21; SH/1			8.2	8.7	8.5
" Vero/1	7	8	7.8	—‡	—
" Vero/2	10	17	6.7	—	—
" Vero/4	16	31	5.9	—	—
" Vero/6	24	47	6.4	—	—
" Vero/8	32	60	7.5	—	—
" Vero/10	40	77	7.5	7.4	7.5

* Original inoculum was a suspension of infected hamster brain.
† Intracerebral inoculation of suckling mice.
‡ Not done.

The inoculated bottles were incubated for one hour at 37°C and washed twice. They were then covered with 5 ml of agar overlay, and were incubated in the dark at 37°C. Plaques were counted on the 10th to 12th day after inoculation, and results expressed as plaque-forming units (PFU).

Tacaribe virus was routinely assayed in 1-day old SM. Mice were inoculated by the intracerebral (IC) route with 0.02 ml of appropriate virus dilution and LD_{50} endpoints calculated with the Reed-Muench formula.

For cytopathology studies, cells were grown on coverglasses in Leighton tubes and stained by Giemsa's method.

Neutralization tests. Immune and pre-immune rabbit sera were heated at 56°C for 30 minutes. Neutralization (N) tests were performed by mixing 0.5 ml of a virus dilution, containing an estimated 100 PFU/0.2 ml, with 0.5 ml of 2-fold serial dilutions of serum. After incubation at room temperature for 1 hour, 0.2 ml of the mixture was inoculated per bottle and the overlay was added as described above. Titers were expressed as the highest serum dilution producing plaque reduction of at least 80% of the simultaneous control assay in a 1:8 dilution of pre-immune or normal rabbit serum. Neutralization tests in tubes, using 100 TCD_{50}, were also performed by the method described above.

Relationship of virus dilution to plaque number. Starting at 10^{-6} dilution of virus stock, 0.2 ml of serial 2-fold virus dilutions were inoculated into each bottle. After 1 hour, bottles were removed from the incubator, washed twice with diluent, and overlaid with agar medium described above.

Results and discussion. Cytopathic effects. Fig. 1 shows CPE produced by Tacaribe virus in Vero cells. Morphological changes can be noted as early as 5 days after inoculation. In general, in the case of tube cultures in a stationary position, the 10^{-5} dilution of the virus produced CPE by the 7th day. Examination of unstained cell cultures under low magnification (72×) revealed focal areas of shrunken and granulated cells surrounded by normal appearing cells (Fig. 1b). Later, the affected cells became detached from the tube wall with formation of macroscopically recognizable clear areas. There was extensive (Fig. 1c) or complete destruction of the cell sheet by the 10th day. The CPE in Vero cell cultures has been an all-or-none phenomenon. Thus, if even a few cells show evidence of morphological changes, the entire culture is eventually destroyed. Examination of infected cells of Giemsa stained cover-glass preparations revealed cytoplasmic changes described by Buckley(1). Polymorphic basophilic inclusions of varying size occurred either near the nuclear membrane or irregularly throughout the cytoplasm (Fig. 1d). The nucleus, however, appeared to be intact. Over a 2-month period 10 serial subcultures of Tacaribe virus were made in Vero cultures, resulting in cumulative final dilution of the original inoculum of 10^{-40} (Table I). Subcultures from infected to new cultures were carried out at 5 to 9 day intervals, and the nature of the CPE was not altered by these passages.

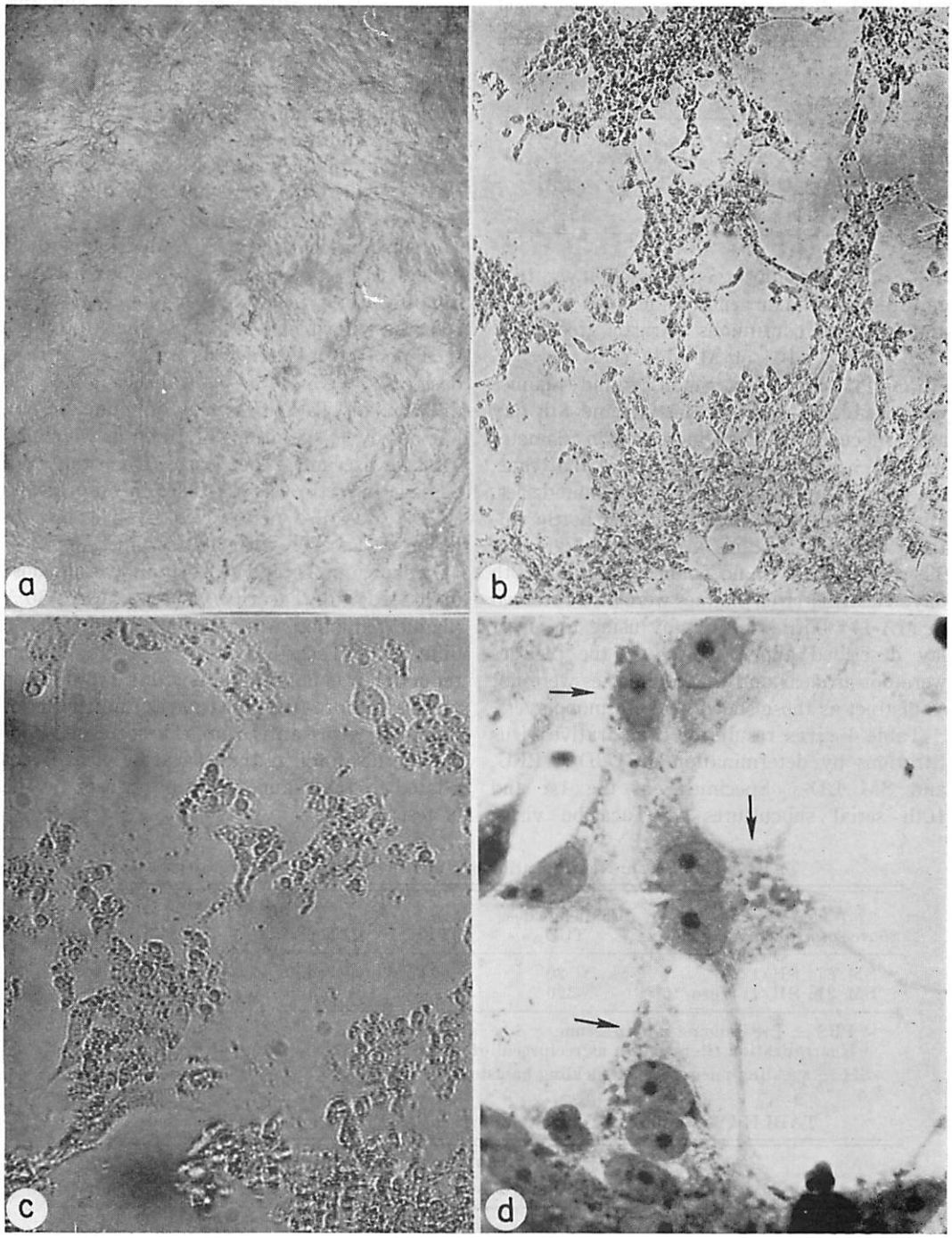

FIG. 1. Cytopathic effects (CPE) produced by Tacaribe virus (TR 11573 strain) in Vero cells. (a) Uninoculated Vero cells, unstained; 72X. (b) Early CPE of Tacaribe virus in Vero cells; day 7 unstained; 72X. (c) Tacaribe virus CPE in later stage in Vero cells; day 10; unstained; 120×. (d) The same as (c) but Giemsa stained; 270×. Note polymorphous basophilic inclusions (arrow) of varying size that occured either near the nuclear membrane or irregularly throughout the cytoplasm.

TABLE II. Relationship Between Plaque Count and Virus Concentration.

Virus dilution (reciprocal)	Plaques/bottle No.	Avg	Log_{10} virus titer (PFU/0.2 ml)
1×10^6	86, 68, 81	78.3	7.8
2×10^6	40, 26, 36	35.3	7.8
4×10^6	17, 15	16	7.8
8×10^6	5, 7	6	7.7
16×10^6	2, 3	2.5	7.6

No CPE was observed in monolayer tube cultures of human embryonic lung (WI-26 and WI-38), continuous African green MK (BSC-1), MA-104 or MA-111 cells.

Plaque production. Small, round plaques were visible as early as the 7th and 8th day after inoculation. They increased in diameter to 0.5-1.5 mm by the 10th day; at that time, the plaques were clear, with sharp boundaries, and were easily counted when the bottle culture was held against a white background. There was little or no change after the 10th day. Tacaribe virus plaques were also obtained in MA-111 bottle cultures by using the overlay described above. However, the plaques were not as clear and the boundaries were not as distinct as those shown in Vero monolayers.

Table I gives results of comparative virus titrations by determination of TCD_{50}, PFU, and SM LD_{50}. Specimens of the 1st and 10th serial subcultures of Tacaribe virus, grown in Vero cells, were assayed, and the titers obtained by various methods were comparable.

The relationship between plaque count and virus concentration is shown in Table II. A linear relationship was observed between the number of plaques and the virus concentration, a result indicating that each plaque was produced by a single virus particle(9).

Neutralization tests. The development of either CPE or plaques by Tacaribe virus was inhibited by type-specific immune rabbit serum. As shown in Table III, when the virus seed pool of infected SH brain was used, a dose of 20 TCD_{50} was neutralized by a 1:150 dilution of Tacaribe virus immune serum, but not by 1:4 dilutions of Junin or Machupo virus antiserum. When virus from the 10th passage in Vero cells was used, a dose of 320 TCD_{50} was also neutralized by a 1:100 dilution of Tacaribe virus antiserum.

As shown in Table IV, when an average dose of 112 PFU of Tacaribe virus was used, 80% plaque reduction was obtained with a 1:256 dilution of Tacaribe virus antiserum. Plaque reduction was not obtained with a 1:8 dilution of normal (pre-immunization), Junin virus or Machupo virus antiserum. These results confirm earlier findings that Tacaribe virus is not related to Junin and Machupo viruses by the N test(3,4).

TABLE III. Serum Neutralization of Tacaribe Virus CPE in Vero Cells.

Source of virus, passage levels	Challenge dose TCD_{50}	PRS*	Rabbit immune serum†		
			Tacaribe	Junin	Machupo
SM/21: SH/1	20	<4	150	<4	<4
SM/21: SH/1: Vero/10	320	—	100	—	—

* PRS = Pre-immune rabbit serum.
† Neutralization titers shown as reciprocal of serum dilution inhibiting CPE.
SM = suckling mice; SH = suckling hamster. — = not done.

TABLE IV. Serum Neutralization of Tacaribe Virus Plaques in Vero Cells.

Antiserum used	Rabbit immune serum dilution								Titer*
	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	
PRS†	112‡	—	—	—	—	—	—	—	<8
Tacaribe	—	—	0	2	9	12	32	64	1:256
Junin	39	67	84	115	—	—	—	—	<8
Machupo	59	91	—	—	—	—	—	—	<8

* Titer shown as reciprocal of serum titer calculated for 80% reduction of plaque counts.
† PRS = Pre-immune rabbit serum.
‡ Average PFU/bottle. — = not done.

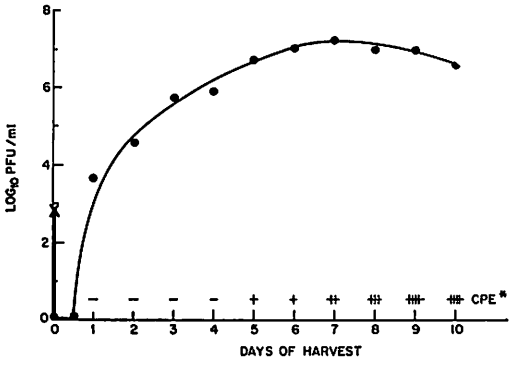

FIG. 2. Gowth curve of Tacaribe virus (TR 11573 strain) in Vero cells. *CPE — = no cellular change, + = 25%, ++ = 50%, +++ = 75%, ++++ = 100% cells affected. X: Virus dose = 9.1 x 10^2 PFU/4.1 X 10^6 cells = 1/4500.

Virus replication. Cell cultures in 2 oz. bottles were washed once and inoculated with 0.2 ml of a 10^{-5} dilution of the stock virus (9.1 $\times$ 10^2 PFU by assay). As there were 4.1 $\times$ 10^6 cells per bottle, this corresponds to an approximate dose of 1 PFU per 4500 cells (0.0002).

The virus inoculum was left in contact for 1 hour at 37°C: the cultures were then washed twice to remove unadsorbed virus and incubated at 37°C after covering with 5 ml of maintenance medium. At daily intervals, 2 bottles were frozen and stored at —70°C. The paired bottles were thawed, pooled and clarified by centrifugation at 2,000 rpm for 15 minutes, and their supernatant fluids were assayed for PFU. Results are shown in Fig. 2. The peak titer of 2.1 $\times$ 10^7 PFU/ml was obtained on day 7, at which time about 50% of the cells showed CPE. After day 7, the virus titer declined for 2 days, while CPE progressed to a complete destruction of the cell monolayer. It has been reported that the rate of Tacaribe virus multiplication in fluid cultures did not correspond to the rate of plaque development in cultures of primary Rhesus MK cells(3), as shown with other arbovirus TC systems. However, in this study, the rate of virus multiplication in fluid cultures did correspond to the rate of plaque development in Vero cells under agar overlay.

Summary. An African green monkey kidney cell line (Vero) has been found to support growth of Tacaribe virus. Cytopathic effects (CPE) were pronounced and appeared within 7 days after inoculation with 10^{-5} dilution of the virus. They were reproducible in 10 serial subcultures. Tacaribe virus also produced distinct plaques with sharp boundaries in monolayer cultures of Vero cells under agar overlay. A linear relationship was observed between plaque counts and virus concentration. The development of CPE and plaques was specifically inhibited by Tacaribe virus immune rabbit serum, but not by Junin or Machupo virus antiserum. Virus assays obtained by titration endpoints of CPE or by plaque counts in tissue culture were comparable to those obtained by titration in suckling mice. When the cultures were infected with <0.001 PFU/cell, virus yields reached their peaks within 7 days.

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LASSA FEVER, A NEW VIRUS DISEASE OF MAN FROM WEST AFRICA

III. ISOLATION AND CHARACTERIZATION OF THE VIRUS*

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ABSTRACT: Fourteen isolates of Lassa virus were recovered in Vero cell cultures from material—serum, pleural fluid, urine, and throat washings—of four cases of Lassa fever. Viremia of 1 to 2 weeks' duration, with TCD_{50} titers ranging from 2 to 4.5 dex per ml, was observed. The agent did not infect the *Aedes aegypti* and *Aedes albopictus* continuous cell lines. When newborn and adult mice were inoculated intracerebrally with Lassa virus, complement-fixing and neutralizing antibodies were detected in their serum; in addition, adult mice showed signs closely resembling those seen in adult mice inoculated with lymphocytic choriomeningitis (LCM) virus. Lassa virus was isolated from urine of infected mice as late as 83 days after inoculation. Multiplication of Lassa virus in Vero cell cultures was not inhibited by the incorporation of 5-bromodeoxyuridine in the medium; hence the virus probably contains ribonucleic acid. The finding that the agent is susceptible to the action of sodium deoxycholate suggests the presence of a lipid-containing envelope. Electron-microscopy studies reveal a spherical shape. Filtration studies indicate a diameter of the virus between 70 and 150 mμ. The 14 isolates, insofar as studied, are indistinguishable from one another. In extensive serologic studies, Lassa virus has been compared with and found distinct from numerous arboviruses and other viruses. By complement-fixation test, it cross-reacts to a low degree with LCM virus, and possibly also with some members of the Tacaribe group.

(Accepted 20 January 1970)

Lassa fever, a severe febrile illness with hemorrhagic manifestations, has been described in four patients, three of them infected in nature in Nigeria[1] and one infected in the laboratory.[2] This paper reports the recovery from these patients of 14 isolates of a hitherto unknown virus, Lassa virus; describes some of the properties of the agent; and presents the results of serologic studies.

MATERIALS AND METHODS

Collection and Inoculation of Materials

The materials examined comprised serum, pleural fluid, urine, and throat washings; details are given in Table 1. Throat washings were collected by having the patient (J.C.) gargle with 1 ml of 0.85% NaCl solution; this material was then added immediately to 5 ml of 7.5% bovine albumin (fraction V) in phosphate-buffered saline solution supplemented with 1,000 units of penicillin and 1,000 μg of streptomycin per ml. All specimens were either inoculated immediately after collection or held at 4°C until transfer to a Revco freezer (−65°C).

Serum specimens, both undiluted and in serial 10-fold dilutions, were inoculated in 0.1-ml amounts into three to 21 replicate cell cultures. In some instances, serum undiluted and in dilution 10^{-1} was also inoculated into mice. The diluent used was 0.75% bovine albumin (fraction V) in phosphate-buffered saline solution, pH 7.2.

Urine specimens, undiluted and in dilution 10^{-1}, were inoculated in 0.1-ml amounts into six replicate cultures. Throat washings, prepared as described, were inoculated in 0.1-ml amounts into 21 replicate cultures.

Animal Inoculations

For reasons of safety, animal inoculations were held to a minimum and done only in mice. The

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TABLE 1
Source of materials, and isolations of Lassa virus

Patient	Sex	Locale of infection	Date of onset, 1969	Outcome of infection	Material, and date collected		Isolation in Vero cells
L.W.	F	Lassa, Nigeria	20 Jan.	Death, day 6	Serum, 26 Jan.	(day 6)	+
C.S.	F	Jos, Nigeria	3 Feb.	Death, day 10	Serum, 6 Feb.	(day 3)	+
					Serum, 13 Feb.	(day 10)	+
L.P.	F	Jos, Nigeria	20 Feb.	Recovery	Serum, 25 Feb.	(day 5)	+
					Serum, 5 March	(day 13)	+
					Pleural fluid, 5 March	(day 13)	+
					Serum, 6 March	(day 14)	+
					Serum, 20 March	(day 28)	0
					Serum, 29 March	(day 37)	0
J.C.	M	New Haven, Conn.	9 June	Recovery*	Serum, 16 June	(day 7)	+
					Serum, 18 June	(day 9)	+
					Urine, 18 June	(day 9)	+
					Throat washing, 18 June	(day 9)	+
					Throat washing, 23 June	(day 14)	+
					Urine, 26 June	(day 17)	+
					Urine, 11 July	(day 32)	+
N.Y.	F	—	In contact with L.P. in Nigeria, Feb. '69		Serum, 26 Feb. '69		0
C.C.	F	—	Stationed in Nigeria		Serum, 23 Sept. '68		0
Controls					Diluent		0

* For complete history of J.C., see Leifer *et al.*[2]

animals used were 1 day or 25 to 30 days old, and came from the Yale Arbovirus Research Unit stock colony derived from Charles River mice. For attempts to propagate the virus, newborn and young adult mice were inoculated intracerebrally (ic) with 0.02 ml and 0.03 ml, respectively; for preparation of immune serum and ascitic fluid, mice were inoculated intraperitoneally. All ic inoculations and bleeding of the mice were done under deep ether anesthesia.

Cell Cultures

The Vero cell line[3] was used at transfer levels 120 to 129. Stock cultures were grown in Roux bottles. At weekly intervals, confluent monolayers were dispersed with a mixture of equal parts of trypsin (0.025%) and Versene (1:5,000). Cells were transferred by a 1:4 split and suspended in 90% Eagle's basal medium (EBM)[4] made up with Hanks's balanced salt solution (HBSS) plus 10% calf serum.

Large Leighton tubes fitted with 3- by 1-inch microscope slides, French square bottles, and 2-oz flint-glass prescription bottles were individually seeded with 10 ml, 10 ml, and 7 ml, respectively, of a cell suspension representing cells collected from one Roux bottle in 320 ml of growth medium. These cultures were used in 4 to 7 days.

Stationary tube cultures were prepared with cell suspensions representing the cells of one Roux bottle harvested in 320 ml of an outgrowth medium consisting of 80% EBM made up with HBSS, 10% tryptose phosphate broth, and 10% fetal-bovine serum; each tube was seeded with 1 ml of cell suspension. These cultures were used after incubation for 2 to 3 days at 37°C.

Before inoculation of cultures in Leighton tubes, French square bottles, and stationary tubes, the respective growth media were replaced with a maintenance medium containing 97% EBM made up with HBSS plus 3% fetal-bovine serum. The maintenance medium was buffered with 0.1 to 0.4 ml of 2 M Trizma®-HCl* per 200 ml of medium. Fluids were changed at weekly intervals.

All media contained 100 units of penicillin and 100 μg of streptomycin per ml except for that

* Sigma Chemical Company, St. Louis, Missouri.

added to urine or throat-washing specimens, in which case the final concentration of antibiotics was increased to 1,000 units of penicillin and 1,000 μg of streptomycin.

During experiments, cultures in fluid media were incubated at 35° to 37°C. Two-oz plaque bottles were incubated at 36°C after overlay of cultures with freshly prepared agar.* The composition of the nutrients in the overlay was as described by Simizu *et al.*[5] except that HBSS was employed instead of Earle's. The final overlay mixture contained 100 μg of diethylaminoethyl-dextran.†

The *Aedes aegypti* and *A. albopictus* continuous cell lines, transfers 92 and 100, respectively, were used as described elsewhere.[6,7]

Cultures used as controls were inoculated with diluent only, but were otherwise treated as described.

Infectivity Titrations

Stationary tube cultures or cultures in 2-oz plaque bottles were inoculated with 0.1-ml volumes of serial 10-fold dilutions of test material, and examined at intervals for appearance of cytopathic effects (CPE) or plaques. Tissue-culture cytopathic dose $(TCD)_{50}$ titers were calculated by the method of Reed and Muench[8] and are expressed in dex[9] per ml.

Cytopathology

Large Leighton tubes with semiconfluent monolayers grown on slides were inoculated with increasing doses (100 to 1,000 TCD_{50}) of Lassa virus, strain L.P. At intervals, slides were fixed in Bouin's fluid for 15 minutes and then extracted with 70% ethyl alcohol until the yellow color was completely removed. Slides were stained overnight in an inverted position in a mixture of 1 ml of Giemsa,‡ 2 ml of phosphate buffer, pH 7.2, and 37 ml of distilled, demineralized water. They were then rinsed in distilled, demineralized water, dried at room temperature, immersed in xylene, mounted in Bioloid mounting medium, and examined under a light microscope.

Electron Microscopy

Electron-microscopy studies of Vero cell cultures infected with Lassa virus are described in the following paper in this series.[10]

Filtration

The method has been described.[11] Ten ml of infected tissue-culture fluid harvested on day 6 after inoculation was added to 30 ml of diluent, and the resulting mixture, designated "original virus," was pipetted into a Millipore casing after removal of 2 ml. The upper inlet of the casing was connected with N_2 in a tank, and the mixture was filtered by pressure (adjusted to 23 psi) successively through Millipore membranes having average pore diameters of 450, 220, and 100 mμ; a prefilter was used in all instances. Two ml of filtrate was removed at each stage; between 15 and 18 ml of fluid remained after final passage through the 100 mμ filter. The entire procedure was conducted at room temperature and took 30 to 40 minutes. Samples of the "original virus" and of the successive filtrates were titrated *in vitro* within the following 40 minutes.

Treatment with Sodium Deoxycholate

The *in vitro* method has been described.[12] The test preparation consisted of equal volumes of infected, undiluted tissue-culture fluid and a 1% solution of sodium deoxycholate; control mixtures were prepared a) with infected, undiluted tissue-culture fluid and diluent, and b) with the chemical and diluent. After incubation of the mixtures for 1 hour at 37°C, they were diluted serially 10-fold, and each dilution was inoculated in 0.1-ml amounts into a set of three cultures. Cultures were examined daily for 6 days for development of CPE.

Nucleic-Acid Determinations

The method has been described.[13] Vero cell cultures were washed three times with HBSS, then fed with 1 ml per culture of maintenance medium containing 10^{-5} M 5-bromodeoxyuridine (BUDR).* Cultures for parallel control titrations were treated identically but were fed maintenance medium without BUDR.

Treatment with Betapropiolactone

The effect of betapropiolactone (BPL) on Lassa virus was studied mainly with a view to

* Ionagar No. 2; Colab Laboratories, Inc., Chicago, Illinois.

† DEAE dextran; Pharmacia, Uppsala, Sweden.

‡ Chroma-Gesellschaft, Schmid and Company, obtained from Roboz Surgical Instrument Company, Inc., Washington, D.C.

* Calbiochem, Los Angeles, California.

determining whether satisfactory but noninfectious complement-fixing (CF) antigens could be prepared. (The authors are indebted to Dr. D. H. Clarke for this study.) Fluids from virus-inoculated and control Vero cell cultures were brought to pH 9 by the addition of 1/10 volume of 1 M Tris buffer, pH 9. BPL, in dilutions of 1%, 2%, and 3% in ice-cold water, was added immediately to culture preparations in the proportion of 1 to 9, to give final BPL concentrations of 0.1%, 0.2%, and 0.3%. After treatment for 24 to 48 hours at refrigerator temperature, the fluids were tested for infectivity *in vitro* and for CF activity.

Antigen Preparation and Serologic Tests

The source material for preparation of CF antigens with Lassa virus was tissue-culture fluid containing cellular debris. The fluid was used either without any treatment or after concentration. Concentrated antigens were prepared as follows: the 1-ml volumes of supernatant fluid from 30 to 160 tubes were pooled, and the pooled fluid was then introduced into dialysis tubing (Fisher, seamless cellulose) of 3/4-inch or 1 1/8-inch diameter, laid on a bed of Acquacide and further covered with Acquacide, and held at room temperature for 3 to 10 or 12 hours, depending on the initial volume and the degree of concentration desired. The concentrated material was then dialyzed overnight at 4°C against 50 to 200 volumes of 0.16 M sodium chloride. Concentrations of five- to 20-fold were thus obtained. Fluids from cultures inoculated with diluent only were treated in the same way and used as control antigens.

CF and hemagglutinating antigens for established viruses, arboviruses and otherwise, were prepared from infected infant-mouse brain tissue by the sucrose-acetone extraction method.[14]

A semi-micro CF test was used.[15] As first attempts showed that unconcentrated fluids from cell cultures infected with Lassa virus had low antigenic titers, when such fluids were used the volume of antigen in a test was raised from the usual one drop to three drops, while the remaining reagents were kept at standard volume. This maneuver seemingly brought about a threefold increase of antigen without any anticomplementary or nonspecific effects of consequence. Concentrated antigens were used in the usual one-drop volume.

For hemagglutination-inhibition (HI) tests,[14] serum specimens were treated with kaolin to remove nonspecific inhibitors.

Neutralization tests were done either in stationary tube cultures by the constant-serum, varying-virus method, or in 2-oz plaque bottles by the plaque-reduction method. Before being tested, serum specimens were inactivated for 30 minutes at 56°C.

RESULTS

Isolation and Properties

As summarized in Table 1, Lassa virus was isolated in Vero cells from each category of material tested—serum, pleural fluid, urine, and throat washing—and as late as day 32 of illness in the case of patient J.C. The occurrence of a laboratory infection with Lassa virus in J.C. was confirmed by the observation that the CPE produced by the isolate from his day-7 serum was specifically inhibited by the day-37 serum from patient L.P. (Case 3).[1] Control cultures appeared normal throughout the experiments.

Observations in cell cultures

Cytopathic changes in Vero cells were similar with the 14 isolates. CPE appeared as early as day 4 after inoculation, at which time the following changes were noted: a) the presence of single necrotic cells throughout the monolayer, along with minute holes caused by the falling out of such cells; b) the occurrence of groups of 10 to 20 rounded, granulated cells localized within focal areas, as well as large, empty spaces left by the detachment of such groups from the glass. During days 5 to 8, most cultures showed rounding and granulation of about 50% of cells, with detachment of about 25% of cells in some cultures and, in a few, severe or complete destruction of the cell sheet. Cellular destruction was accompanied by increased acid production, as indicated by conversion of the phenol red indicator in the maintenance medium to bright yellow.

To the extent attempted, second and subsequent passages of the isolates were readily carried out. Titration endpoints (Table 2) were usually reached within 7 days after inoculation.

The isolate obtained from the day-5 serum of patient L.P. was selected as the tentative prototype and designated strain L.P. A stock of this strain was prepared as follows: 80 stationary tube cultures were inoculated with 0.1-ml amounts of

TABLE 2
Titers of Lassa virus isolates in Vero cells

Isolate	Passage level	TCD_{50} titer, dex/ml
L.W., serum, day 6	1st	4.5 or >
	2nd	6.2
C.S., serum, day 3	1st	3.0
	2nd	6.2
C. S., serum, day 10	1st	2.6
	2nd	6.7
L.P., serum, day 5	1st	2.0
	2nd	7.3
L.P., serum, day 13	1st	3.5
	2nd	6.5
L.P., pleural fluid, day 13	1st	4.5 or >
	2nd	6.2
L.P., serum, day 14	1st	4.0
	3rd	7.5
J.C., serum, day 7	1st	3.5
	2nd	7.5 or >
J.C., serum, day 9	1st	4.0
J.C., urine, day 9	1st	1.5
	2nd	6.5
J.C., throat washing, day 9	1st	2.3
	2nd	4.3
J.C., throat washing, day 14	1st	2.0
J.C., urine, day 17	1st	1.7
	2nd	3.5
J.C., urine, day 32	1st	1.0
	2nd	4.5 or >

a 10^{-3} dilution of first-passage tissue-culture fluid. Five days later, fluids from these cultures were harvested, dispensed in 1-ml amounts into screw-cap tubes, shell-frozen, and stored in the Revco freezer (–65°C). This stock preparation had a TCD_{50} titer of 7.5 dex per ml. Distinct plaques, 2 mm in diameter, were observed in 2-oz plaque-bottle cultures of Vero cells overlaid with agar containing nutrients and neutral red. Plaque production during days 5 to 8 was reproducible, and assays of plaque-forming units were comparable to assays in tube cultures. In a qualitative plaque-reduction neutralization test, plaque formation was specifically inhibited by the day-28 serum of patient L.P.

Strain L.P. did not infect either the *A. aegypti* or *A. albopictus* cell line.

Infectivity for mice

Twenty newborn mice, in two litters, were inoculated ic with the day-14 serum of patient L.P., undiluted and in dilution 10^{-1}. One mouse was found dead on day 9 after inoculation; the rest survived, and Lassa virus was isolated from

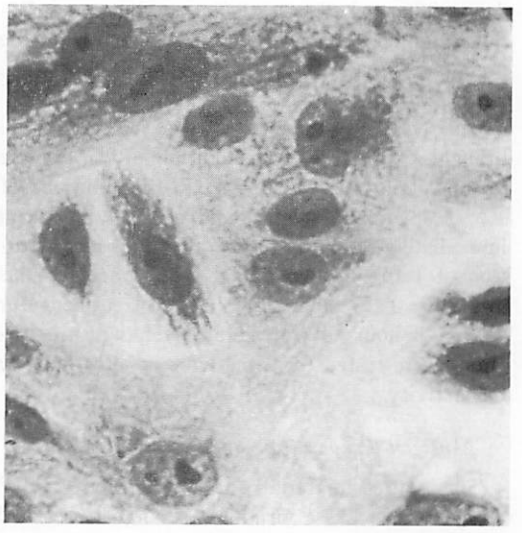

FIGURE 1. Vero cells harvested 5 days after inoculation with diluent. Note pale-staining cytoplasm. (Stained by Giemsa's method, pH 7.2; × 400.)

their urine on day 83 after inoculation. These survivors were later used as a source of antiserum. When brain tissue from the dead mouse, in dilution 10^{-2}, was passed to two new litters of newborn mice, none of the animals showed signs of illness; these mice were bled 30 days after inoculation for possible source of antibodies. As pooled serum samples from the surviving mice of each passage have repeatedly given CF titers between 1:64 and 1:256, as well as 80% plaque reduction in a neutralization test, it appears that the virus was propagated through two passages in newborn mice.

In a second experiment, newborn and adult mice were inoculated with 2 dex of strain L.P. of the virus from third-passage tissue-culture fluids harvested on days 3 and 6 (see Table 8). None of the 26 newborn mice inoculated with tissue-culture material harvested on day 3, either undiluted or in dilution 10^{-1}, showed signs of illness for as long as any were observed (60 days); however, Lassa virus was isolated from their urine on day 46 after inoculation.

All of the adult mice inoculated with tissue-culture material harvested on day 3 remained well through day 5 after inoculation. On day 6, five of eight given undiluted material and six of seven given dilution 10^{-1} were found dead. When two other mice given undiluted material

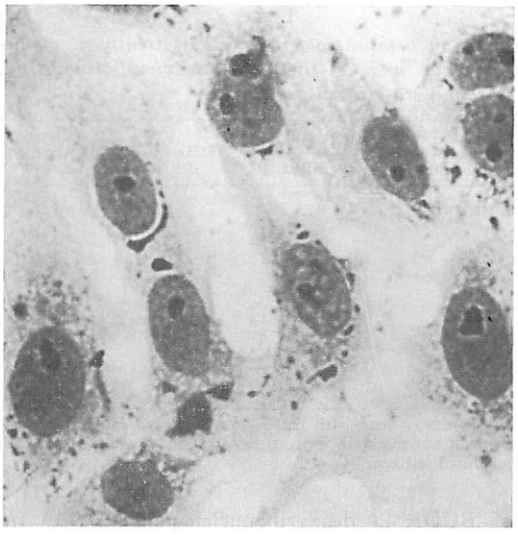

FIGURE 2. Vero cells harvested 5 days after inoculation with 300 TCD_{50} of Lassa virus, strain L.P. Note pleomorphic, basophilic cytoplasmic aggregates; few cells detached from glass. (Stained by Giemsa's method, pH 7.2; × 400.)

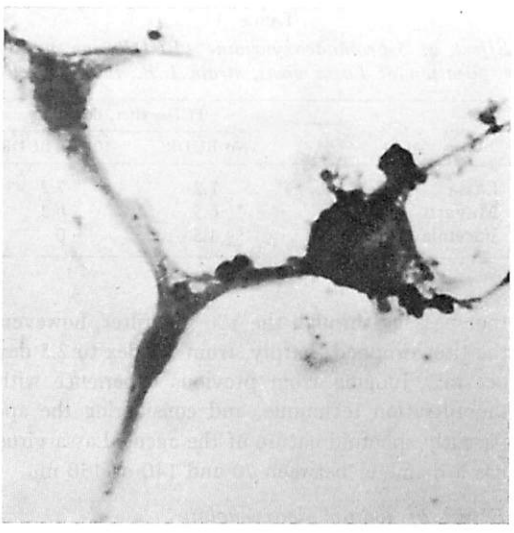

FIGURE 3. Vero cells harvested 8 days after inoculation with 300 TCD_{50} of Lassa virus, strain L.P. Note severe destruction of cells; a few pyknotic cells attached to glass. (Stained by Giemsa's method, pH 7.2; × 400.)

were spun by their tails, they had convulsions, temporarily stopped breathing, and had rigidly extended hind legs; a fine tremor could be felt when they were held by the tail. These signs closely resemble those seen in adult mice inoculated with lymphocytic choriomeningitis (LCM) virus and may well be an indication of choriomeningitis, not necessarily due to LCM virus.

With the tissue-culture material harvested on day 6, again all of the newborn mice inoculated with undiluted material (18) or dilution 10^{-1} (nine) remained healthy for as long as any were kept under observation. Of the adult mice, one of seven given undiluted material was found dead on day 9 after inoculation, and two of eight given dilution 10^{-1} were found dead on day 7.

In the urine studies reported above, although CF antibodies to the virus were present in the serum of both mouse groups at the time the urine was collected, each sample of urine had a virus TCD_{50} titer in Vero cells of 3.5 dex per ml. The two isolates were identified in CF test.

Cytopathology

Vero cell cultures inoculated with strain L.P. of the virus and stained with Giemsa showed basophilic pleomorphic aggregates localized in the cytoplasm of single cells or in cells grouped together in foci (Figs. 1 and 2). Ultimately, severe cellular destruction occurred (Fig. 3).

Electron microscopy

Studies of pelleted cells of Vero cultures infected with strain L.P. of the virus revealed the occasional presence of spheroid particles similar in morphology to those described for LCM virus.[16] Such particles were not found in control preparations. Details are reported in the following paper in this series.[10]

Determination of size

Inoculation of samples of the successive Lassa virus filtrates into Vero cells showed little loss of titer in the 220 mμ filtrate when compared with the original material (Table 3). After fur-

TABLE 3

Titers of Lassa virus, strain L.P., after successive filtration through Millipore membranes of decreasing average pore diameter (APD)

Virus suspension	TCD_{50} titer, dex/ml
Original, unfiltered	6.7
Filtered through 450 mμ APD membrane	7.2
Filtered through 220 mμ APD membrane	5.7
Filtered through 100 mμ APD membrane	2.5

TABLE 4

Effect of 5-bromodeoxyuridine (BUDR) on multiplication of Lassa virus, strain L.P., in Vero cells

Virus	TCD_{50} titer, dex/ml	
	No BUDR	10^{-5} M BUDR
Lassa	7.2	7.2
Mayaro	6.5	6.2
Vaccinia	4.5	0

ther passage through the 100 mμ filter, however, the titer dropped sharply, from 5.7 dex to 2.5 dex per ml. Judging from previous experience with the filtration technique, and considering the apparently spheroid nature of the agent, Lassa virus has a diameter between 70 and 140 or 150 mμ.

Effect of sodium deoxycholate

After treatment with sodium deoxycholate, Lassa virus did not produce CPE in Vero cells. The TCD_{50} titer of virus in the control mixture (infected tissue-culture fluid and diluent) was 7.5 dex per ml.

Nucleic-acid studies

As shown in Table 4, the TCD_{50} titer of strain L.P. of the virus was unchanged in the presence of BUDR, a deoxyribonucleic-acid (DNA) inhibitor. Similar results were obtained with Mayaro, a known ribonucleic acid (RNA)-containing virus, whereas BUDR suppressed the development of CPE by vaccinia virus, a DNA-containing agent.

TABLE 5

Effect of betapropiolactone (BPL) treatment on infectivity and CF titer of Lassa virus, strain L.P., from Vero-cell culture fluid

Tissue-culture fluid		TCD_{50} titer, dex/ml	Titer as antigen in CF test*
Inoculum	BPL (%)		
Lassa virus	0	6.5	1:8
	0.1	0	1:8
	0.2	0	1:8
	0.3	0	1:8
Diluent	0	0	0
	0.1	0	0
	0.2	0	0
	0.3	0	0

* Test was done with day-37 serum of patient L.P.; three drops of antigen were used. 0, no fixation of complement with undiluted antigen.

Development of a CF System

The results in Table 5 demonstrate that satisfactory but noninfectious CF antigens can be

TABLE 6

CF test: development of antibodies following infection with Lassa virus, and effect of volume of antigen on degree of fixation of complement

Antigen*			Serum, patient L.P.									
			Day-14 serum; dilution, 1:				Day-28 serum; dilution, 1:					
Type	Number of drops	Dilution, 1:	8	16	32	64	8	16	32	64	128	256
Lassa, strain L.P.	1	1	0†	0	0	0	0	2	1	1	0	0
		2	0	0	0	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0	0	0	0
		8	0	0	0	0	0	0	0	0	0	0
Control	1	1	0	0	0	0	0	0	0	0		
		2	0	0	0	0	0	0	0	0		
		4	0	0	0	0	0	0	0	0		
		8	0	0	0	0	0	0	0	0		
Lassa, strain L.P.	3	1	0	0	0	0	4	4	4	4		
		2	0	0	0	0	3	4	4	4		
		4	0	0	0	0	0	2	2	1		
		8	0	0	0	0	0	0	0	0		
Control	3	1	0	0	0	0	0	0	0	0		
		2	0	0	0	0	0	0	0	0		
		4	0	0	0	0	0	0	0	0		
		8	0	0	0	0	0	0	0	0		

* Vero-cell culture fluid.

† 0, no fixation; 4, complete fixation. Test was done with two units of complement.

TABLE 7

CF test: effect of concentration through evaporation on titer of tissue-culture antigen

Antigen (3 drops)		Serum	
Virus	Concentration	L.P., day 37	L.D., normal
Lassa, strain L.P.	None	64/8*	0
	7X	64/64	0
Control	None	0	0
	7X	0	0

* Numerator, reciprocal of serum titer; denominator, reciprocal of antigen titer. 0, no reaction at dilution 1:8 of serum and undiluted antigen.

prepared with Lassa virus. Treatment with even 0.1% BPL reduced the TCD_{50} titer of infected tissue-culture fluid from 6.5 dex per ml to 0. Neither this nor greater concentrations of BPL, however, reduced the fluid's antigenic titer in CF test, nor did nonspecific reactions occur.

The effect of increasing the amount of Lassa virus antigen in a test by augmenting the volume used is shown in Table 6. Whereas only traces of fixation were observed with one drop of undiluted antigen, clear-cut fixation was obtained with three drops. The specificity of this test was controlled by use of the virus-positive, day-14 serum from patient L.P. and of an antigen preparation from uninfected tissue-culture fluid. Overnight titration of complement showed that its titer was not affected by use of three drops of antigen.

The effect of increasing the amount of Lassa virus antigen in a test by concentration is shown in Table 7. The titer of the antigen with the day-37 serum of patient L.P. increased from 1:8 to 1:64, while the control serum, L.D., did not react with the antigen. No reactions occurred with the control antigen, nor did concentration result in anticomplementariness on overnight titration of complement.

Table 8 shows the growth curve and development of Lassa CF antigen in Vero cells. This study was made with 80 Vero cell cultures inoculated with strain L.P. from second-passage tissue-culture fluid in dilution 10^{-5}; 80 other cultures inoculated with diluent only served as controls. Beginning with time 0 and ending with day 9 after inoculation, eight infected and eight control tubes were removed daily and held at −60°C. The combined fluid and cell phases of each set of eight cultures were subsequently pooled and

TABLE 8

Growth curve of Lassa virus, strain L.P., in Vero cell cultures and development of CF antigen

			CF test	
Type	Day of harvest	TCD_{50}, dex/ml	Antigen titer, 1:	Remarks
Infected	0	0	1*	AC†
Control		0	1	AC
Infected	1	4.5	1	AC
Control		0	1	AC
Infected	2	5.7	1	AC
Control		0	1	AC
Infected	3	7.5	8	AC, undiluted
Control		0	1	AC
Infected	4	8.2	16	AC, undiluted
Control		0	1	AC
Infected	5	8.5	16	AC, undiluted
Control		0	1	AC
Infected	6	7.5	16	
Control		0	0	
Infected	7	6.7	16	
Control		0	0	
Infected	8	6.7	16	
Control		0	0	
Infected	9	6.2	8	
Control		0	0	

* Reaction with serum of patient L.P., titer 1:128. 0, no fixation with undiluted antigen, three drops.
† AC, antigen anticomplementary on overnight incubation.

assayed simultaneously for infectivity and for presence of CF antigen. As shown in the table, TCD_{50} titers of 7.5 dex per ml were seen as early as day 3. Peak titers of 8.2 and 8.5 dex were obtained on days 4 and 5; thereafter, as cellular damage increased, titers decreased, reaching 6.2 dex per ml on day 9.

CF-test results were closely parallel. Through day 2, when TCD_{50} titers were either 0 or less than 6 dex per ml, antigen was undetectable. On day 3 it appeared in fair titer, 1:8; the titer rose to 1:16 on day 4, where it remained until day 9, when it dropped back to 1:8. The slight anticomplementary action noted on day 3 and a few subsequent days with antigen used undiluted did not interfere with the test. Interestingly, the antigens that were anticomplementary during the first 5 days subsequently lost this activity, so that titers of 1:16 or 1:8 on days 6 through 9 could be considered more legitimate than the same titers obtained on earlier days. The development of superior CF antigens for Lassa virus thus

TABLE 9

CF-test relations of 11 isolates of Lassa virus

Test no.	Antigen (3 drops)	Serum, L.P., day 37
1	L.W., serum, day 6	128/8*
	C.S., serum, day 3	128/16
	C.S., serum, day 10	128/16
	L.P., serum, day 5	128/8
	L.P., serum, day 13	128/16
	L.P., pleural fluid, day 13	128/8
	L.P., serum, day 14	128/8
	Control	0
2	J.C., serum, day 7	64/8
	J.C., serum, day 9	64/8
	J.C., urine, day 9	64/2
	J.C., throat washing, day 9	64/2
	Control	0

* Numerator, reciprocal of serum titer; denominator, reciprocal of antigen titer. 0, no fixation with undiluted antigen.

appears to coincide with severe cellular destruction.

Serologic Identification

Similarity of isolates

Thus far, 11 of the Lassa virus isolates have been tested against the day-37 serum from patient L. P. in CF test. As shown in Table 9, these 11 fixed complement at similar titer. As the isolation studies were carried out at a time when the only antiserum specimens available were from patient L.P., it is not yet possible to state with certainty that all 14 isolates are identical.

Search for antigenic relations between Lassa and other viruses

HI tests were done with samples of the day-37 serum from patient L.P. and of pooled serum from a group of mice that survived inoculation as newborns with strain L.P. of Lassa virus; both serum specimens had CF-antibody titers of 1:128. When these serum samples, in dilutions beginning at 1:10, were tested against eight units each of antigens for a number of known arboviruses, the only reaction occurred with yellow fever antigen (titer of 1:20), and this was undoubtedly the result of the patient's having been vaccinated previously with 17D yellow fever vaccine. The other antigens used in the test were prepared with chikungunya, o'nyong-nyong, Semliki, and Sindbis viruses of group A; dengue types 1, 2, 3, and 4, Banzi, Ntaya, Spondweni, Wesselsbron, West Nile, and Zika of group B; and Bunyamwera, Germiston, Bwamba, and Ťahyňa from other groups.

An extensive search was made by CF test for possible cross-reactions between strain L.P. of Lassa virus and a large number of established agents, arboviruses and otherwise. No cross-reactions were observed when an antigen for strain L.P., with a homologous titer of 1:16, was screened undiluted and in dilution 1:4 against dilutions, beginning at 1:8, of polyvalent or monovalent immune-mouse serum or ascitic fluid for the following groups or individual viruses: group A (range of titers with homologous antigens, 1:32 to 1:256), group B (1:16 to 1:256), group C (1:64 and 1:128), Congo (1:256), epizootic hemorrhagic disease of deer, New Jersey (1:16), Marburg (1:64), Nairobi sheep disease (1:128), Piry (1:64), Rift Valley fever (1:256), simian hemorrhagic fever (1:256), and the Tacaribe group (1:64 to 1:256).

Results were also negative when the day-28 and day-37 serum specimens from patient L.P., with homologous titers of 1:64 to 1:128, were tested in dilutions beginning at 1:8 against dilutions 1:4 and 1:8 of antigens for the following agents: Acará, Akabane, Amapari, Anopheles A, Anopheles B, Aruac, Bahig, BeAn 67949, BeAn 84381, BeAn 141106, Bertioga, Bhanja, Boraceia, Bunyamwera, Bushbush, Cali 874, California, Capím, Chaco, Chandipura, Changuinola, CoAr 3319, CoAr 3627, Cocal, Colorado tick fever, Congo, Cotia, EgAn 1477-61, EgAn 1825-61, EgAn 3782-62, Embu, encephalomyocarditis of mice, epizootic hemorrhagic disease of deer (New Jersey), EthAr 1846-64, Farallon, Flanders, Guajará, Guamá, Guaroa, Germiston, Hart Park, hepatoencephalitis of mice, herpes, Hughes, IbAn 2898, IbAn 10065, IbAn 15736, IbAn 17143, IbAn 17854, IbAn 20433, IbH 11306, Ieri, IPD/A 401, Irituia, J-19, J-134, Johnston Atoll, Junín, Jurona, Kamese, Kemerovo, Kern Canyon, Ketapang, Koongol, Kwatta, LCM, Lagos bat, Lone Star, Lukuni, Machupo, Marburg, Marco, Microtus 1056, Mirím, Mossuril, Mount Elgon bat, Newcastle disease, Nyamanini, Nyando, Omsk hemorrhagic fever, Oropouche, Piry, phlebotomus Neapolitan, phlebotomus Sicilian, polioencephalitis of mice, poxvirus (ectromelia), Pacuí, Pichinde, Punta Toro, rabies, SA-Ar 136, Sathuperi, Sawgrass, Silverwater, simian hemorrhagic fever, Soldado, SudAr 1169-64, SudAr 1275-64, Tacaribe, Tacaiuma,

TABLE 10
*Cross-CF test relations of Lassa, LCM, and Tacaribe group viruses**

Antigen	Serum or ascitic fluid: Lassa, human: L.P., day 37	Lassa, human: J.C., day 52	Lassa, mouse: 1 (1 inj.)	Lassa, mouse: 2 (2 inj.)	Lassa, mouse: 3 (2 inj.)	Lassa, mouse: 4 (2 inj.)	Lassa, mouse: 5 (2 inj.)	Lassa, mouse: 6 (3 inj.)	LCM, mouse: 1 (3 inj.)	LCM, mouse: 2 (3 inj.)
Lassa, L.P.	128/16†	32/32	128/16	256+/32	256/16	32/16	256/16	128/4+	8/8	8/4
LCM	0	0	4/4	4/4	16/32	0	16/32	0	256/256	128+/128+
Amapari	0				4/16	0	16/32	4/16		
Junín	0				4/16	2/4	8/8			
Pichinde	0				0	0	0			
Tacaribe	0				4/8	0	4/16	0		
Tamiami	0				0		2/8			
Controls:										
1. (Akabane)	0				2/4	0	0	0		
2. (Ilhéus)	0				4/16	0	0	0		
3. (Lipovnik)	0				2/8	0	0	0		
4. (Normal tissue)	0	0	0	0	2/4	0	0	0	4/4	0

* First dilution of serum: 1:4 for human, 1:2 for mouse; first dilution of antigen: 1:2 for Lassa, 1:4 for all others.
† Numerator, reciprocal of serum titer; denominator, reciprocal of antigen titer. 0, no fixation at lowest dilution of serum used.

Tamiami, Tataguine, Tembé, Thogoto, Triniti, Turlock, vesicular stomatitis-Indiana, vesicular stomatitis-New Jersey, Wad Medani, Witwatersrand, yellow fever, and YM 31. With few exceptions, all antigens reacted with their homologous serum samples in the same tests. (The authors are indebted to Dr. Robert E. Shope for testing the great majority of these antigens.)*

Because of the similarity noted between Lassa and LCM viruses in terms of behavior in newborn and adult mice and the image observed by electron microscopy, further CF tests were carried out to investigate their possible relation more thoroughly. These tests were done mainly with use of serum and ascitic fluid from mice repeatedly inoculated with a virus; however, serum from mice immunized with Lassa virus was also used as it became available. The existence of a low, but often reproducible, degree of cross-reactivity between the Lassa and LCM systems was soon apparent. In view of the reported morphologic resemblance between LCM virus and Machupo virus of the Tacaribe group,[17] observations were also expanded to include members of that group. The results obtained thus far are given in Table 10. (The authors are indebted to Dr. Robert E. Shope for some of the data reported in the table.)

Serum from the two patients that survived the infection repeatedly did not react with antigen for LCM or for Tacaribe group viruses, even at dilution 1:4 of serum and antigen. Immune-mouse serum or ascitic fluid, however, gave a different result, particularly when multiple-injection, high-titered reagents were used. Although in some instances a slight degree of nonspecificity was shown by the immune reagents, on the whole there seems little doubt but that a cross-reaction exists between Lassa and LCM viruses, and possibly also between Lassa and members of the Tacaribe group.*

* A series of tests with the day-14 and day-28 serum specimens from patient L.P. was carried out by Dr. Morris Schaeffer, director, Bureau of Laboratories, City of New York, with the following antigens: influenza A, parainfluenza 1, 2, and 3, adenoviruses, *Mycoplasma pneumoniae*, psittacosis-lymphogranuloma venereum, Q fever, LCM, Eastern equine encephalomyelitis, Western equine encephalomyelitis, St. Louis encephalitis, rickettsialpox, typhus, Rocky Mountain spotted fever, herpes, proteus OX19, and proteus OX2. Either antibodies were not detected or they were present at low titers and the same in the two serum specimens.

* The authors thank Dr. Richard W. Emmons, Department of Health Laboratories, California, and Dr. Wallace P. Rowe, National Institutes of Health, Bethesda, Maryland, for data showing a relation between Lassa and LCM viruses by the fluorescent-antibody technique.

Serologic survey

Serum from 70 American missionaries and members of their families who had been stationed in Nigeria between 1966 and 1969 were tested against Lassa virus in plaque-reduction neutralization and CF tests. Two of the serum samples had neutralizing antibodies in low titer, and one of the two reacted by CF test with a titer of 1:8.

DISCUSSION

There is no epidemiologic evidence to indicate that Lassa virus is an arthropod-borne virus. No data exist to support the hypothesis that in nature it is biologically transmitted by a bloodsucking arthropod. Rather, the epidemiologic information available in connection with the four cases of Lassa fever described[1,2] indicates either personal contact; aerosol infection, as suggested by isolation of the virus from throat washings; or contact with contaminated fomites, as suggested by finding of the virus in urine of patient J.C. and of laboratory-infected mice.

A commonly used technique for isolation of arboviruses, ic inoculation of infant mice, was not successful with Lassa virus. The day-14 serum of patient L.P. thus inoculated did not induce infection in 19 of 20 mice; a single mouse was found dead on day 9 after inoculation. It was later shown that most adult mice inoculated ic died. Virus-containing preparations readily infected Vero cell cultures but did not infect the *A. aegypti* and *A. albopictus* continuous cell lines. Since isolates were readily recovered from serial specimens of three of the patients, it was decided not to attempt reisolation from specimens determined to be virus-positive. This decision was taken in view of the hazards associated with working with the virus.

As pointed out by Frame *et al.*, the agent apparently was stable while suspended in whole serum at low temperature.[1] Thus, isolations were made from four serum specimens which, after being stored frozen initially, were kept over ice in an insulated container during 4 days' transit from Jos, Nigeria, to New York.

In patients L.W. and C.S., viremia was present up to the day of autopsy. From patient L.P., who recovered, virus was isolated from serum 5, 13, and 14 days after onset; thus viremia, with TCD_{50} titers ranging from 2 to 4.5 dex per ml, existed over a period of 2 weeks. From patient J.C., infected in the laboratory and treated with immune plasma,[2] virus could not be isolated from the blood 2, 3, 5, and 8 days after the administration of immune plasma: TCD_{50} titers of 3.5 and 4 dex per ml dropped to zero after such treatment. As reported by Leifer *et al.*, the donor's convalescent-phase serum, with a CF titer of 1:32 to 1:64 at the time of collection, was apparently instrumental in modifying the course of the acute systemic illness.[2]

Although the virus could not be isolated from the blood of J.C. on four occasions subsequent to the administration of immune plasma, it was recovered from a throat washing collected 5 days after such treatment and from urine specimens collected 8 and 23 days after treatment. Specimens of these same materials taken on days 26 and 43 after treatment were virus negative. (See Table 1, Leifer *et al.*[2])

On the grounds of the known properties of its virion, and assuming that additional electron-microscopy studies fail to provide evidence of helical symmetry, Lassa virus appears to fit in the taxon designated Togavirus, which has been proposed by a study group of the International Committee on Nomenclature of Viruses.*

The number of cases of Lassa fever studied and the number of survey serum samples tested for antibodies are too small to permit any inferences as to the prevalence of Lassa virus in the Nigerian population.

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MORPHOLOGICAL IDENTIFICATION OF THE AGENT OF KOREAN HAEMORRHAGIC FEVER (HANTAAN VIRUS) AS A MEMBER OF THE BUNYAVIRIDAE

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Summary Korean haemorrhagic fever (KHF) (Hantaan virus), a rodent-borne viral illness, is an important cause of human disease throughout much of Asia and Eastern Europe. The agent responsible for KHF has not yet been conclusively identified. Plaque-purified KHF virus was concentrated and then banded in a potassium tartrate gradient. Material from the 1·17–1·19 g/ml band was examined by electron microscopy and particles with a morphology identical to that of the family Bunyaviridae were found. The particles were aggregated by KHF serum but not by saline solution or non-immune serum. Identification of KHF virus as a member of the family Bunyaviridae implies a potential for spread by arthropod vectors.

Introduction

Korean haemorrhagic fever (KHF) was described several decades ago and is known by many names throughout Asia and Europe.[1] It is a severe, not uncommon disease, found in a geographic area from Japan to Europe which is occupied by about one-half of the world's population. The agent was isolated in 1978 by Lee et al.[2] and was grown in tissue culture by French in 1981.[3] The virus has not, however, been satisfactorily purified for morphological identification. We describe the purification and morphological characteristics of Hantaan virus.

The strain of Hantaan virus used for this study, designated 76-118, has been registered in the Working Catalogue of Arthropod-Borne Viruses.[4] Since it is a direct descendent of the 76-118 isolate described by Lee et al. in the original isolation of the virus,[2] and by French for growth in A-549 cells,[3] it has a well-defined pedigree. It is also 1 of the 3 strains studied by Lee and Cho in their effort to characterise the virus.[4]

Several important characteristics of the 76-118 strain have been identified. (a) It was initially isolated from the rodent *Apodemus agrarius corae*.[2] (b) Antibody titres to 76-118 strain rise 4-fold or more in persons with typical KHF illness.[2,3] (c) Antibody titres to strain 76-118 have been found in sera from patients with nephropathia epidemica in Scandinavia and epidemic haemorrhagic fever with renal syndrome in China and Japan.[5–8] (d) Strain 76-118 is sensitive to lipid solvents and is acid labile.[3]

Methods

Virus Cultivation

A fifth A-549 cell passage of strain 76-118 virus was inoculated into 30 mm roller tubes of E-6 cells, a cloned line of Vero cells maintained at the Centers for Disease Control (CDC) and available from the American Type Culture Collection. These infected E-6 cells were maintained in minimal essential medium (MEM) with 10% fetal calf serum (FCS) and virus was harvested after 12 days. Six further passages were made in 75 cm^2 (surface) flasks by inoculation at a multiplicity of infection of approximately 1 tissue culture infective dose ($TCID_{50}$) per cell.

Virus Measurement and Neutralisation

Virus titres were determined as both $TCID_{50}$ and plaque-forming units (PFU). $TCID_{50}$ was determined by making serial 10-fold dilutions and determining the highest dilution at which virus replicating in E-6 cells could be detected by immunofluorescence assay (IFA). A plaque assay was performed both for the purpose of cloning and for measuring the virus stock titre in PFU. Virus was inoculated into 25 cm^2 flasks containing E-6 cells and, after a 30 min adsorpton, 3 ml of basal minimal Eagle's (BME) medium containing 1% agarose, 0·03 mol/l HEPES, 4% FCS, and antibiotics were added to each flask. 10 days after infection the E-6 cells were overlayed with an additional 2 ml of the same medium. 3 days later, 2 ml of fresh BME medium containing 8·25 mg/dl of neutral red was added to each flask. 18 days after infection, plaques with a diameter of approximately 4 mm were easily detectable.

For a plaque reduction neutralisation test, 0·1 ml of undiluted, 1:10, and 1:100 dilutions of convalescent serum (700047) were mixed with 0·1 ml volumes of serial 20-fold dilutions and undiluted stock virus from a stock containing 4×10^3 PFU/ml. Serum-virus mixtures were incubated at 37°C for 1 h and then added to E-6 cells. The plaque assay was performed as described above.

Preparation for Morphological Study

To prepare purified virus for morphological studies, infected E-6 cells were harvested on day 12, pelleted, resuspended in 'Tris'-NaCl-ethylene diaminetetraacetic acid (TNE), frozen in dry ice, and thawed. This material was combined with the supernatant from the infected cells, and this mixture was clarified and precipitated in polyethylene glycol (PEG)-NaCl overnight. A virus pellet was obtained from the PEG-NaCl by centrifugation at 10 000 *g* for 30 min. The pellet was resuspended in TNE and layered on a 0–50% potassium tartrate and 30–0% glycerol gradient and centrifuged to equilibrium at 30 000 rpm for 18 h in a Spinco 40 rotor. Virus appeared as a light-scattering band at a density of 1·17–1·19 g/ml. The band was collected and the virus was freed of gradient salts by centrifugation at 30 000 rpm for 1 h. The virus was resuspended in 300 µl of TNE buffer for immunoelectron microscopy (IEM). Three 50 µl volumes of material were mixed with (1) 50 µl of a 1:50 dilution of normal serum, or (2) 50 µl of a 1:50 dilution of convalescent serum, or (3) phosphate-buffered saline, pH 7·2. Each sample was incubated at 37°C for 1 h and then treated with an equal volume of 1·0% buffered glutaraldehyde before removal from the maximum containment laboratory. The virus was prepared for electron microscopy by the pseudoreplica technique[9] and particles were stained with 2·5% phosphotungstate (PTA), pH 6·5.

Plaque-purified virus was obtained by picking a single plaque at 10^{-7} dilution from seventh-passage material which had been concentrated by pelleting on to a 70% sucrose cushion at 30 000 rpm for 1 h. The plaque was picked, diluted in MEM with 10% FCS, and grown in E-6 cells. Media were replenished on these cells 10 days after infection and virus was harvested from cells and supernatant on day 13. These virus harvests were inoculated undiluted into 25 cm^2 flasks and then into 150 cm^2 flasks containing E-6 cells to produce virus pools for experimental use.

The 10^{-7} dilution plaque was passaged 4 times before inoculation into 150 cm^2 roller bottles. Fifteen roller bottles were infected and 11 days later the supernatants were pooled, clarified, and precipitated with PEG. The resulting virus pellet was layered on a potassium tartrate glycerol gradient and centrifuged. The resulting visible virus band was removed from the gradient, pelleted, and resuspended in 200 µl of TNE. 50 µl of this virus suspension was mixed with 50 µl of 1% glutaraldehyde, stained, and examined by electron microscopy.

Serology

A seventh E-6 passage of Hantaan virus was inoculated into 30 150 cm^2 roller bottles. The cells were harvested by means of glass beads after 12 days' incubation at 37°C. Drops of cell suspension

were placed on 'Teflon'-coated 12-well slides, dried, and the slides were placed in acetone for 10 min. After irradiation (5×10^5 rads) the slides were tested by IFA. IFA tests were performed on infected cells by means of convalescent-phase plasma (700047) from a KHF patient. This patient had had a typical disease course and a >4-fold rise to Hantaan virus measured by IFA performed with infected *Apodemus* lung and on tissue-culture-grown virus.[2,3] Serum 700047 did not contain antibody to types 1, 2, or 3 reovirus or to Lassa, Marburg, Ebola, Congo-Crimean, or Rift Valley fever viruses as determined by IFA.

Six pairs of coded acute and convalescent phase sera from patients with acute KHF were tested against the unpurified virus and the plaque-purified virus to detect rises in antibody titre.

Fig. 1—Korean haemorrhagic fever virus agglutinated by KHF convalescent-phase serum.
Bar = 100 nm.

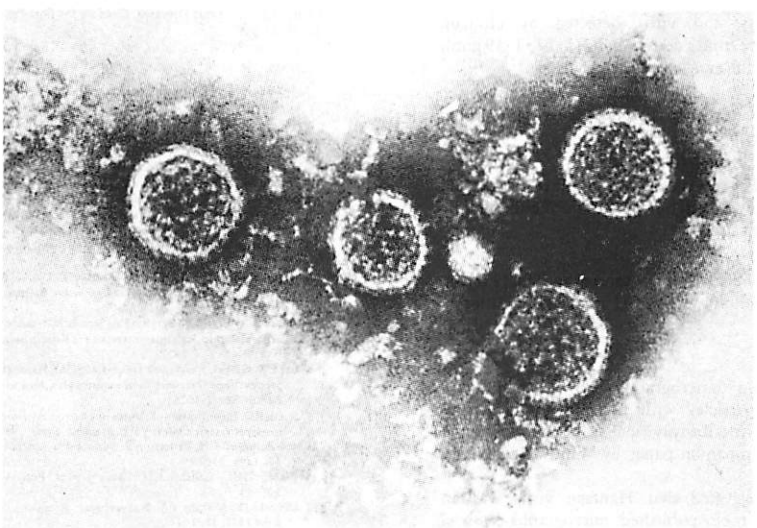

Fig. 2—Korean haemorrhagic fever virus in PBS.

Particles are relatively homogenous in appearance, having a limited membrane, and a morphology similar to the Uukuniemi group of viruses. Surface projections are clearly visible around periphery of viruses. Bar = 100 nm.

Results

KHF convalescent-phase serum agglutinated the virus into large clumps of 10 to more than 100 particles (fig. 1), whereas normal serum and phosphate-buffered saline controls contained mostly free-lying particles (fig. 2). The particles are roughly spherical and have a unit membrane envelope. They range from 80 to 115 nm in diameter, with the average diameter being 92·5 nm. Subunits are visible around the periphery of the virus and in some instances, the subunits can be seen on the surface of the virus giving it a knobby appearance. Although the surface projections are regularly arranged, no definite symmetry could be assigned to their arrangement. These particles are homogeneous in appearance and very similar in morphology to the Uukuniemi group of viruses[10] which make up the genus Uukuniemi of the Bunyaviridae, but which are antigenically unrelated to the genus *Bunyavirus*.[11]

4-fold rises in antibody titre were demonstrated by IFA in all six pairs of sera from typical KHF patients against the unpurified antigen and the plaque-purified antigen. Compared with control serum, convalescent immune serum (700047) at a dilution of 1:100 reduced plaque formation by 10^3 PFU.

90% or more of E-6 cells harvested 11–15 days after infection contained viral antigen by IFA. The antigen did not bind antibodies to type 1, 2, or 3 reovirus nor antibodies to numerous haemorrhagic fever viruses including Lassa, Ebola, Marburg, Congo Crimean haemorrhagic fever and Rift Valley fever viruses.

Discussion

Evidence that the virus identified in this study represents the agent of Korean hemorrhagic fever is as follows:

1. The virus is agglutinated by KHF convalescent serum but not by control serum.

2. Paired sera from persons with typical KHF demonstrated fourfold rises in antibody titres to the unpurified virus and the plaque-purified virus.

3. The infectivity and virus detected by electron microscopy both come from a density band (1·17–1·19 g/ml) which is the same as for most other Bunyaviridae.[12]

4. The average size of the virus accords with that reported for the Bunyaviridae.[12]

5. We found no IFA evidence of the presence of a second virus, specifically reovirus, in our preparations either before or after plaque purification..

6. Compared with normal control serum the 700047 serum reduces plaque formation by 10^3 PFU at a serum dilution of $\geqslant$1:100.

Our negative-stain micrographs show unequivocal evidence of virus particles with morphological features identical to those of the Bunyaviridae. This observation is supported by the companion paper by White et al. in this issue.[13]

Lee and Cho suggested that Hantaan virus was an orbivirus, although their published micrographs show a reovirus.[4] We and others[3] have found that Hantaan virus is sensitive to lipid solvents. Lee[14] found this as well, but this is not a characteristic of reoviruses (or orbiviruses), suggesting that two agents may have been present in Lee and Cho's tissue culture systems. One agent detected by IFA was probably Hantaan virus sensitive to solvent, while the other, which appeared to be a reovirus by electron microscopy would not have been sensitive. We have found that about 90% of the infectious antigen is destroyed after 30 min at 56°C (McCormick JB, Sasso DR, unpublished). The fact that before fixation Lee and Cho inactivated their preparation at 56°C for 30 min and then observed a reo-like virus which would be expected to resist heating, again suggests the two viruses were present. They did not discuss the recovery of immunologically functional Hantaan antigen after heating.

We had difficulty in purifying the virus from cellular material alone despite detection of cellular viral antigen at infectivity levels of 10^6 $TCID_{50}$ and by IFA. We found that the virus was more easily purified from supernatant material.

The morphology of the Bunyaviridae has not been systematically studied so that we cannot compare the morphology of Hantaan virus with many of the Bunyaviridae. Nevertheless, the Uukuniemi genus has ordered surface projections[12,15] whereas the *Bunyavirus* genus does not. The thick membrane and regular surface arrays of Hantaan virus resemble those of Uukuniemi virus. Serological testing so far has failed to show that the Hantaan and Uukuniemi viruses have antigens in common (French GR, Lee HW, unpublished data). Assignment of Hantaan virus to a definite serogroup depends on further serological and biochemical data.

Two other haemorrhagic fever viruses are found among the Bunyariridae, namely Congo Crimean haemorrhagic fever virus and Rift Valley fever virus. These viruses belong in separate serogroups and are generally transmitted by arthropods. This suggests that an arthropod vector of Hantaan virus should be sought.

Limited quantities of inactivated antigen made from our plaqued, purified virus will be made available to those interested in performing serological studies. In addition, the plaque-purified strain 76-118 will be placed in the American Type Culture Collection.

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Preliminary Communications

ISOLATION AND PARTIAL CHARACTERISATION OF A NEW VIRUS CAUSING ACUTE HÆMORRHAGIC FEVER IN ZAIRE

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AN outbreak of hæmorrhagic fever with an exceptionally high mortality-rate occurred in southern Sudan and northern Zaire with peak case-rates in September, 1976. A W.H.O. International Commission operated in Sudan and Zaire from October onward.[1,2] Blood and tissue specimens from persons with hæmorrhagic disease were sent to laboratories in Belgium and England, and findings from these laboratories appear in the accompanying reports.[3,4] While these specimens were being studied, Mr E. T. W. Bowen (Microbiological Research Establishment, Porton Down) sent an aliquot of an acute blood specimen from a patient in Zaire (no. 718, patient M.E.) to the Center for Disease Control, Atlanta, for additional study.

This specimen, and all subsequent acute specimens, were inoculated into Vero (African green monkey) cells. Three days later a distinct cytopathic change (focal rounding and refractility) was evident, and an aliquot of supernatant fluid was removed for negative contrast electron microscopy.

ELECTRON MICROSCOPY OF CELL CULTURES

Carbon-coated grids were sequentially floated on droplets of the cell-culture fluid and then on 2% sodium silicotungstate pH 7. Large numbers of filamentous virus particles were seen (fig. 1). They were approximately 100 nm in diameter and varied in length from 300 nm to more than 1500 nm. Many had terminal blebs. Particles had regular surface projections approximately 10 nm long, and when stained they were seen to have internal cross-striations indicative of a helical core structure (fig. 2). In all details, these particles were indistinguishable from Marburg virus particles studied in 1967 (isolates from Germany) and 1975 (isolate from South Africa).[5-7] Two characteristics were more prominent in the 1976 Zaire isolate: there was more branching of the filamentous particles (fig. 1); and more evidence of envelope continuation beyond the ends of the more rigid internal structure (fig. 1, arrow).

Vero cells infected with the same isolate from Zaire were examined also by thin-section electron microscopy. Filamentous virus particles were found budding from the plasma membrane of cells (fig. 3), and many of the cells contained inclusion bodies. These intracytoplasmic inclusions were complex and distinct, and consisted of a finely fibrillar or granular ground substance which condensed into tubular structures. The latter have been considered to be the internal helical structure of mature virus particles. These tubules were sectioned randomly, some in cross-section, some linearly. The virus particles in these sections were identical to those observed in the 1967 and 1975 isolates.[6,8]

POSTMORTEM LIVER SPECIMENS

Evidence of infection was seen by light microscopy in three postmortem human liver specimens from Zaire (received in formalin). Infection of two of these was confirmed by electron microscopy. Focal eosinophilic hepatocellular necrosis with modest inflammatory infiltration was prominent. Large eosinophilic inclusions were present in many intact hepatocytes, especially near sites of severe necrosis (fig. 4). These rather smooth and refractile inclusions were so characteristic that they have diagnostic significance. Plastic-embedded formalin-

TABLE I—COMPARISON OF RECIPROCAL I.F.A. TITRES OF MARBURG ('67, '75) AND MARBURG-LIKE ('76) VIRUS DISEASE SERA

Year of illness	Country	Human sera	Time after onset	Antigen	
				Marburg '67	no. 718 '76
1967	Germany	U	5 mo.	128	<10
		K	5 mo.	64	<8
1975	South Africa	DO	1 mo.	64	<4
		MC	4 mo.	64	<4
1976	Sudan	no. 8	±12 days	<2	16
		no. 9	±12 days	<2	<2
	Zaire	no. 5	1 mo.	4	160

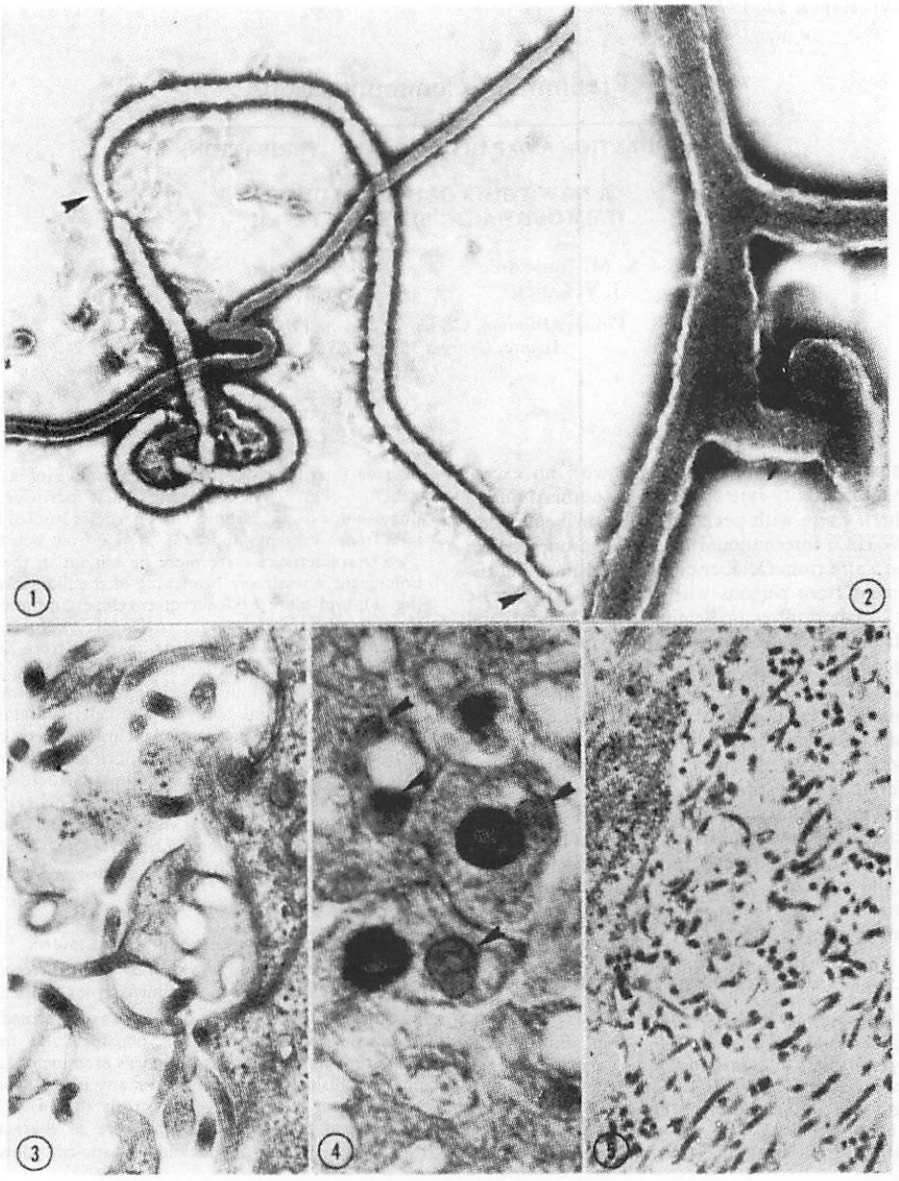

Fig. 1—Ebola virus in first Vero-cell passage after inoculation with patient's blood from Zaire (reduced from × 44 000).

Some filamentous particles in negative contrast preparations were more than 1500 nm long; particles had a uniform diameter of 100 nm and some had ragged ends (arrows).

Fig. 2—Virus particle penetrated by negative contrast medium showing internal cross-striations (reduced from × 115 000).

Fig. 3—Virus particles, in ultrathin section, budding from plasma membrane of Vero cell at 3 days after infection (reduced from × 49 000).

All morphological characteristics were similar to those of Marburg virus as studied in 1967 and 1975.

Fig. 4—Liver from fatal case in Zaire (reduced from × 1100).

Large eosinophilic inclusion bodies (arrows) in many hepatocytes. Focal necrosis and inflammation. Formalin fixation, hæmatoxylin and eosin.

Fig. 5—Virus particles in distended extracellular space in liver from fatal case in Zaire (reduced from × 20 000).

Despite poor tissue preservation, massive numbers of virus particles and characteristic inclusion bodies were identified by electron microscopy of two of three necropsy specimens.

TABLE II—COMPARISON OF I.F.A. TITRES OF GUINEAPIGS IMMUNISED (SINGLE INJECTION) AGAINST MARBURG ('67) AND MARBURG-LIKE ('76) VIRUSES

Immunising agent	Guineapig sera	Days after inoculation	I.F.A. titres with antigen of:	
			Marburg '67	no. 718 '76
Marburg '67	G.P. 16	12	>640	5
Marburg-like '76	G.P. 1	10	2	256

fixed necropsy specimens were examined with the electron microscope. Although preservation of liver tissue was poor, large numbers of filamentous virus particles and inclusion bodies (masses of tubules) were found (fig. 5) which were indistinguishable from those in Marburg virus-infected human and guineapig livers studied in 1967 and 1975.[6-8]

ANTIGENIC COMPARISON WITH MARBURG

An antigenic difference between this isolate and Marburg '67 was demonstrated by indirect immunofluorescence (I.F.A.). An infected Vero-cell suspension was placed in drops on slides, air dried, and then acetone-fixed for 10 min at room temperature. Slides were stored at −70°C until tested. Marburg '67 antigen slides, prepared in like manner, were used for comparison. Reciprocal titres obtained with convalescent human sera drawn during the 1967, 1975, and 1976 outbreaks are listed in table I. With the exception of a weak reaction to Marburg antigen at a 1/4 dilution of the Zaire convalescent serum, the new isolate was distinct from Marburg virus. The homologous Marburg titres of 128 and 64 obtained with '67 and '75 antigens and antisera were comparable to those reported by Wulff and Conrad.[9]

A single-injection immune serum to the new agent was prepared in guineapigs, and reciprocal I.F.A. tests were performed with available similar reagents for Marburg virus. Reciprocal titres (table II) further confirmed the distinctness of the two viruses. Although one of two early convalescent sera from Sudan gave a positive reaction with the Zaire antigen (table I) further work is needed to determine whether the hæmorrhagic-disease agents from the two countries are identical.

EBOLA VIRUS

With the concurrence of Prof. S. R. Pattyn, Institute of Tropical Medicine, Antwerp, and Mr E. T. W. Bowen, Microbiological Research Establishment, Porton Down, the name Ebola virus is proposed for this new agent. Ebola is a small river in Zaire which flows westward, north of Yambuku, the village of origin of the patient from whom the first isolate was obtained. In deference to the countries involved and to the lack of specific knowledge of the original natural source of the virus, it is also suggested that no names of countries or specific towns be used.

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Rubella Virus Contains One Capsid Protein and Three Envelope Glycoproteins, E1, E2a, and E2b

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We have analyzed the structure of rubella virus proteins labeled metabolically with [^{35}S]methionine, [^{3}H]mannose, and [^{3}H]glucosamine or externally with [^{3}H]borohydride after galactose oxidase treatment. Four structural proteins, with M_rs of about 58,000 (E1), 47,000 (E2a), 42,000 (E2b), and 33,000 (C), were resolved on sodium dodecyl sulfate-polyacrylamide gels. Tryptic peptide maps obtained from [^{35}S]methionine-labeled proteins indicated that E1 and C were unrelated to each other and to E2a and E2b, whereas the latter two gave similar, if not identical, maps. E1, E2a, and E2b were associated with the envelope and were located externally on the virus particle, whereas the C protein was associated with the RNA in the nucleocapsid. Solubilization of the virus with Triton X-100, followed by removal of the nucleocapsid and the detergent, resulted in the formation of soluble envelope protein complexes (rosettes) containing E1, E2a, and E2b. Although external labeling with [^{3}H]borohydride and metabolic labeling with [^{3}H]glucosamine suggested that all three proteins were glycosylated, only E1 and E2b were efficiently labeled with [^{3}H]mannose. It is thus possible that the difference in migration between E2a and E2b is due to differences in glycosylation. Analysis by immunoprecipitation and sodium dodecyl sulfate-gel electrophoresis of intracellular [^{35}S]methionine-labeled structural proteins synthesized in the presence and absence of tunicamycin supported the conclusion that E1 and E2 are glycoproteins. Unglycosylated E1 and E2 had an M_r of about 53,000 and 30,000, respectively.

Rubella virus (RV), a member of the genus *Rubivirus* within the Togaviridae family, consists of a lipoprotein envelope membrane and a nucleocapsid core (26). Although the structural proteins of RV have been the subject of biochemical studies for more than 10 years, there is still uncertainty as to their number, size, and properties. Three to four major polypeptides and four to five minor ones have been identified in purified rubella virions by several investigators (2, 5, 11, 12, 17, 21, 36–39). The apparent molecular weights of the major polypeptides range from 16,500 to 63,000, and those for the minor ones range from 10,000 to 88,000. One of the major polypeptides (M_r = 30,000 to 35,000) appears to be associated with the single-stranded RNA genome in the nucleocapsid. The largest major polypeptide (M_r 55,000 to 63,000) appears to be glycosylated and associated with the viral envelope. (5, 26, 37). In addition, one or two polypeptides with an M_r of about 42,000 to 50,000 present in smaller molar amounts than the large glycoprotein seem to be associated with the envelope as well (37). Whether the minor polypeptide species indeed are virus specific or host cell contaminants has remained unclear.

In this report, we present evidence that highly purified RV contains three glycosylated, membrane-associated proteins, E1 (M_r = 58,000), E2a (M_r = 47,000), and E2b (M_r = 42,000), and one unglycosylated nucleocapsid protein, C (M_r = 33,000). E2a and E2b yielded similar, if not identical, tryptic peptide maps as analyzed both by reverse-phase high-performance liquid chromatography (HPLC) and on cellulose thin-layer plates.

MATERIALS AND METHODS

Virus and cells. The Therien strain of RV, originally obtained from Ann Schluederberg, Yale University, New Haven, Conn., was grown in B-Vero cells in 2-liter glass roller bottles in the presence of Eagle minimum essential medium (MEM) supplemented with 0.2% bovine serum albumin (BSA: Sigma Chemi-

cal Co., St. Louis, Mo.) and with 100 U of penicillin per ml, 50 μg of streptomycin per ml, and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2).

Plaque assay, cloning, and preparation of stock virus. The plaque assay was carried out as described for Uukuniemi virus (24) by using confluent monolayers of B-Vero cells grown on plastic dishes (20 cm^2).

To clone RV, individual plaques were picked with a Pasteur pipette through the agar overlay. Each plaque was suspended in 1 ml of MEM plus 0.2% BSA. The suspension was diluted in a 10-fold series, and the cloning procedure was repeated three times. After the last cloning, stock virus was cultivated from a single plaque by two passages in B-Vero cells. The multiplicity of infection in the second passage was 0.5 PFU per cell. At 24 h after infection, the medium was replaced with a fresh one and the stock virus was harvested twice with 12-h intervals. The final stock virus had a titer of 2×10^8 PFU/ml and a hemagglutination titer of 128.

Virus cultivation, concentration, and purification. B-Vero cells grown in roller bottles were infected with 5 PFU per cell. After adsorption for 2 h at 37°C, cells were washed twice with Hanks salt solution. A 50-ml amount of MEM containing 0.2% BSA and 20 mM HEPES (pH 7.2) was added to each bottle. At 15 h postinfection, the medium was replaced with 30 ml of fresh medium, after which the virus was harvested three times at 8- to 12-h intervals by replacing the medium. The last harvest was done before the appearance of cytopathic effect.

Culture medium, 2.5 to 3.0 liters, was cleared by centrifugation at 10,000 × *g* for 20 min at 4°C. The virus was concentrated from the supernatant with a CH4A concentrator (type H1; Amicon Corp., Lexington, Mass.) to a volume of about 200 ml. After low-speed clarification, the virus was further concentrated by centrifugation through a 3-ml 30% (wt/wt) sucrose cushion made in TN buffer (0.1 M NaCl, 50 mM Tris [pH 7.4]) in an SW27 rotor at 24,000 rpm for 3.5 h at 4°C. The pellet was eluted at 0°C overnight in 200 μl of TNE buffer (0.1 M NaCl, 50 mM Tris [pH 7.4], 1 mM EDTA) per tube. After gentle suspension, the virus (2 to 3 ml) was layered on a sucrose gradient containing, from the bottom, 14 ml of a 30 to 60% (wt/wt) sucrose gradient and 18 ml of a 10 to 25% (wt/wt) sucrose gradient in TN. Centrifugation was for 4 h at 25,000 rpm at 4°C in an SW27 rotor. To localize the virus, 1-ml fractions were collected from below and assayed for hemagglutination activity. The virus-containing peak fractions were pooled, and the virus was concentrated by centrifugation at 25,000 rpm for 2.5 h at 4°C in an SW27 rotor. The virus pellet was resuspended in 200 μl of TN buffer.

Radioactive virus. To obtain radioactively labeled virus, RV was cultivated as described above. At 15 h postinfection, the medium was replaced with 10 or 20 ml of MEM containing 0.2% BSA. When the virus was labeled with [^{35}S]methionine, [^{3}H]mannose, [^{3}H]glucosamine, or $^{32}P_i$, the medium was changed at 15 h after infection and replaced with medium lacking either methionine, glucose, or P_i, respectively. For labeling, 50 μCi of [^{35}S]methionine (1,455 Ci/mmol) per ml, 50 μCi of [^{3}H]mannose (18 Ci/mmol) per ml, or 0.5 mCi of carrier-free $^{32}P_i$ per ml (all from The Radiochemical Centre, Amersham, England) was added. The virus was harvested with 12-h intervals. After the first harvest, the radioactive medium was replaced with unlabeled medium. The virus was purified as described above.

For external labeling, 100 μg of purified virus was incubated with neuraminidase (Behringwerke AG, Hamburg, Germany) and then labeled by the galactose oxidase procedure with [^{3}H]borohydride as described by Gahmberg and Hakomori (7). The labeled virus was concentrated by centrifugation at 25,000 rpm for 1.5 h and 4°C in an SW50 rotor and finally suspended in 50 μl of gel electrophoresis sample buffer.

Isolation of envelope protein complexes. A 100-μg amount of purified unlabeled RV and 10^6 cpm of [^{35}S]methionine-labeled RV were mixed, solubilized with 2% Triton X-100, and fractionated in a 15 to 30% sucrose gradient as described above for the isolation of nucleocapsids. The top fractions containing the envelope glycoproteins were dialyzed against 10 mM Tris-hydrochloride (pH 7.4), lyophilized, and suspended in one-fourth the original volume of water containing 0.5% Triton X-100. The sample was layered on a sucrose gradient containing, from the bottom, 10 ml of a 20 to 50% (wt/wt) gradient made in TN buffer and 15% sucrose made in TN buffer containing 1% Triton X-100 (9). Centrifugation was for 20 h at 39,000 rpm and 20°C in an SW41 rotor. Fractions (0.4 ml each) were collected from below and assayed for radioactivity.

SDS-polyacrylamide gel electrophoresis. Electrophoresis in vertical gradient slab gels in the presence of sodium dodecyl sulfate (SDS) was by the method of Laemmli (15). The acrylamide concentration was 3.3% in the stacking gel and 5 to 16% in the separating gel. The samples were denatured and reduced with 4% SDS and 10% β-mercaptoethanol in the sample buffer and boiled for 3 min. After electrophoresis, the gels containing radioactively labeled material were treated for fluorography and autoradiographed (4) with Kodak RP X-Omat films. The ^{14}C-methylated molecular weight markers used were myosin (M_r = 200,000), bovine serum albumin (M_r = 69,000), ovalbumin (M_r = 46,000), carbonic anhydrase (M_r = 30,000), and lysozyme (M_r = 14,300) (all from Amersham). Gels containing unlabeled proteins were stained with Coomassie blue.

Preparation of antiserum. Antiserum against purified RV was prepared in rabbits by injecting 200 μg of SDS-disrupted virus in Freund complete adjuvant. Three booster injections of 200 μg each were given subcutaneously at 3-week intervals in Freund incomplete adjuvant. Rabbits were bled before immunization (control serum) and after the last injection, and the sera were analyzed for the presence of antibodies against RV by the immunoblotting method and the hemagglutination test. The titer of the immune serum used was 640 as measured with the hamagglutination inhibition test.

Preparation of cytoplasmic extracts. B-Vero cells in 94-mm dishes (55 cm^2) were infected with 5 PFU per cell. After a 2-h adsorption at 37°C, the cells were washed twice with Hanks salt solution. A 5-ml amount of MEM containing 0.2% BSA and 20 mM HEPES (pH 7.2) was added. At 24 h postinfection, the medium was replaced with 5 ml of medium lacking methionine.

Tunicamycin (1 μg/ml; Sigma Chemical Co.) or monensin (10 μM; Calbiochem, La Jolla, Calif.) was added 3 h before labeling. One dish received no drugs and served as a control. For labeling the proteins, 50 μCi of [^{35}S]methionine (1,350 Ci/mmol; Amersham) per ml was added to the medium. After 1 h, the cells were washed twice with MEM containing a 20-fold excess of the normal amount of methionine and incubated in this medium for 30 min. Cells were then washed once with cold phosphate-buffered saline, solubilized in 1 ml of NET buffer (1% Nonidet P-40, 0.4 M NaCl, 0.05 M Tris [pH 8.0], 0.005 M EDTA) containing 100 IU of Trasylol (Bayer, Leverkusen, West Germany), and kept on ice for 10 min. The extracts were centrifuged at 12,800 × *g* for 10 min, and the supernatants were used for immunoprecipitation (22). Noninfected and infected cells labeled in the absence of tunicamycin and monensin served as controls.

Immunoprecipitation. For immunoprecipitation, about 4 × 10^6 cpm of each cytoplasmic extract was diluted into 1 ml of NET buffer containing 100 IU of Trasylol. A total of 5 μl of immuneserum was added, and the mixtures were incubated at room temperature for 1 h. Protein A Sepharose (CL-4B; Pharmacia Fine Chemicals, Uppsala, Sweden) was added, and the samples were incubated overnight at 4°C. The precipitates were concentrated by centrifugation and washed twice with NET buffer and once with 62.5 mM Tris-hydrochloride (pH 6.8) containing 20% glycerol. Samples were then treated with electrophoresis sample buffer, and the supernatants were analyzed by SDS-gel electrophoresis as described above.

Preparation of proteins for peptide analysis. [^{35}S]methionine-labeled proteins for peptide analysis were separated by preparative SDS-polyacrylamide slab gel electrophoresis as described above. After electrophoresis, the 1-mm-thick gel was vacuum dried on a sheet of wetted dialysis membrane, and the radiolabeled proteins were detected by autoradiography for 15 h. Parts of the dried gel containing the labeled proteins were cut out and introduced into 10 ml of 50 mM *N*-ethylmorpholine acetate buffer (pH 8.8) for 10 min. The proteins were then electrophoretically eluted from the gel pieces and concentrated to 200 μl in an Isco model 1750 electrophoretic sample concentrator (1) by using a 50 mM-100 mM *N*-ethylmorpholine acetate (pH 8.8) buffer system (3 W, 4 h, 20°C). Human immunoglobulin G (75 μg) was added to each concentrated sample, and the proteins were precipitated with trichloroacetic acid (20%) at 0°C. The precipitates were washed once with ethanol (−20°C) and once with ethanol-ether (1:1, vol/vol, −20°C) and lyophilized.

The proteins were then oxidized in 150 μl of chilled performic acid (3), lyophilized, and treated with 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin in 400 μl of 50 mM NH_4HCO_3 for 4 h at 37°C. A further 5 μg of trypsin was added, digestion was continued for 4 h, and the digest was lyophilized. Lyophilization was repeated three times after the addition of 1 ml of water each time to the dried samples. The digests were finally dissolved in 200 μl of electrophoresis buffer (acetic acid-pyridine-water, 7:20:970, by volume) and centrifuged (20,000 × *g*, 15 min), and the supernatants were carefully aspirated. A 50-μl sample of each supernatant was separately lyophilized for HPLC analysis. The rest of each supernatant was lyophilized for analysis on thin-layer plates.

Tryptic peptide analysis. For HPLC analysis, the lyophilized samples were dissolved in 60 μl of 0.09% trifluoroacetic acid and 20-μl portions of each sample were analyzed on three different reverse-phase HPLC columns (μBondapak, 3.9 by 300 mm, Waters Associates, Inc., Milford, Mass; Spherisorb S5-phenyl, 4.5 by 250 mm, Phase Separations Ltd., Queensferryl, England; and Vydac 201TP, 4.5 by 250 mm, The Separations Group, Hesperia, Calif.). For chromatography, a 5020 chromatograph (equipped with a variable-wavelength monitor; Varian Associates, Palo Alto, Calif.) was used. The peptides were eluted at a flow rate of 1.0 ml/min with an increasing linear gradient of acetonitrile (0 to 63% in 60 min) in 0.09% trifluoroacetic acid. Elution of radiolabeled peptides was monitored by collecting 0.5-ml fractions and determining their radioactivity in a liquid scintillation counter. Elution of unlabeled carrier peptides was continuously monitored by adsorption at 210 nm as a control.

For analysis on cellulose thin-layer plates (20 by 20 cm; E. Merck AG, Darmstadt, West Germany), the lyophilized samples were dissolved in 2 μl of 0.1 M NH_3, spotted on the wetted plates, and subjected to electrophoresis in acetic acid-pyridine-water (7:20:970, by volume) at 300 V (10 mA) for 75 min. Ascending chromatography was performed in *m*-butanol–acetic acid–water–pyridine (15:3:12:10, by volume) for 5 h. Radiolabeled peptides were detected by autoradiography for 2 to 7 days.

Other methods. Treatment of whole virions with endo-*N*-acetyl-β-D-glucosaminidase H (endoglycosidase H; *Streptomyces griseus*; Seikagaku, Tokyo, Japan) was as described before (23). Protein concentrations were determined by the method of Lowry et al. (18). The density of sucrose gradient fractions was determined by a refractive index, which was converted to its corresponding density. Radioactivity was determined in a xylene–Triton X-100 scintillation cocktail by using a Wallac 81,000 scintillation counter. Sedimentation rates were calculated by the method of Martin and Ames (20). Electrophoretic transfer of proteins to nitrocellulose sheets for immunoblotting analysis was carried out as described by Towbin et al. (34). For electron microscopy, purified RV or glycoprotein complexes were negatively stained with 2% potassium phosphotungstate at pH 7.0 as described before (25).

RESULTS

Purification of RV. Biochemical characterization of RV has been hampered by the relatively low yields of infectious virus, causing problems in obtaining sufficient amounts of virus free of host cell contaminants. To maximize the yield of infectious virus, we prepared a high-titer stock virus from a single plaque obtained after three consecutive plaque purification steps. This stock virus had an infectivity titer of 2 × 10^8 PFU/ml and a hemagglutination titer of 128. For virus purification, the medium was harvested every 12 h for three successive times. To avoid contamination with cell debris, the final harvest

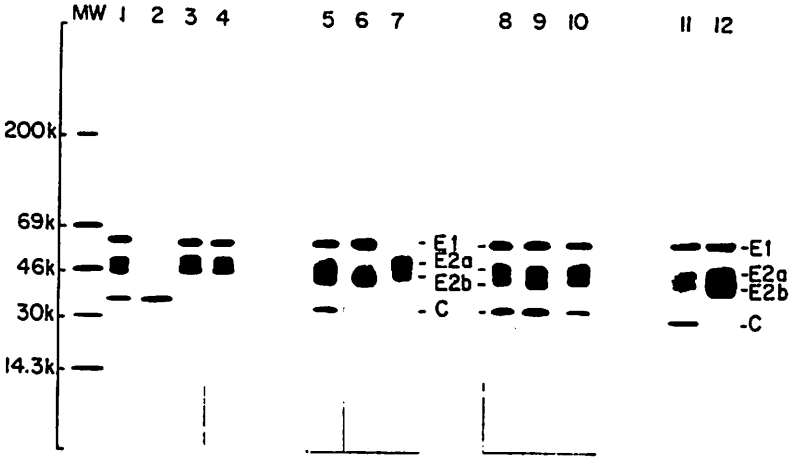

FIG. 1. SDS-gel electrophoresis of RV proteins. Purified virus, labeled either metabolically with [^{35}S]methionine, [^{3}H]glucosamine, or [^{3}H]mannose or externally with [^{3}H]borohydride after treatment with neuraminidase and galactose oxidase, was disrupted with SDS and analyzed on 5 to 16% polyacrylamide gradient slab gels. Lanes 1 to 4, 5 to 7, 8 to 10, and 11 to 12 show the results of four different analyses. (Lanes 1, 5, 8, and 11) [^{35}S]methionine-labeled whole virus; (lanes 2 and 3) [^{35}S]methionine-labeled nucleocapsid and envelope, respectively; (lane 4) [^{35}S]methionine-labeled envelope glycoprotein complexes (rosettes) from fraction 16 in Fig. 6; (lanes 6 and 7) RV labeled with [^{3}H]mannose and [^{3}H]borohydride, respectively; (lanes 9 and 10) [^{35}S]methionine-labeled virus after treatment with neuraminidase and endoglycosidase H, respectively; (lane 12) virus labeled with [^{3}H]glucosamine. The positions of the molecular weight markers in thousands are shown on the left.

was carried out before the appearance of a moderate cell damage. If the harvest was continued to the point of extensive cytopathic effect, the final purified virus preparation regularly contained a large number of contaminating host cell proteins. For purification of RV, we used a simple concentration and purification procedure previously adopted in our laboratory for Semliki Forest virus (SFV) (29) and Uukuniemi virus (25), two other enveloped RNA viruses (see above). In the combined velocity-density sucrose gradient, viral infectivity and hemagglutination activity coincided with the [^{35}S]methionine used to label the viral structural proteins (data not shown). In one typical experiment, 30% of the original infectivity was recovered in the final purified virus preparation.

Analysis of the structural proteins by SDS-gel electrophoresis. Purified [^{35}S]methionine-labeled virus was analyzed by SDS-gel electrophoresis. Four major bands having apparent molecular weights of about 58,000, 47,000, 42,000, and 33,000 were identified (Fig. 1, lane 1). The same protein bands were identified by Coomassie blue staining of unlabeled viral proteins (data not shown). Based on their structure and location in the virion (see below), we have designated these proteins E1, E2a, E2b, and C, using the nomenclature adopted for the alphaviruses (13). The distribution of the [^{35}S]methionine label in E1, E2a, E2b, and C was about 37, 21, 21, and 21%, respectively. Occasionally, two minor bands with apparent molecular weights of about 109,000 and 96,000 were seen. To determine which of the proteins are glycosylated, RV was grown in the presence of either [^{3}H]glucosamine or [^{3}H]mannose. E1, E2a, and E2b were labeled with [^{3}H]glucosamine (Fig. 1, lane 12). In contrast, only E1 and E2b were efficiently labeled with [^{3}H]mannose (Fig. 1, lane 6). These results suggest that E1, E2a, and E2b are glycoproteins and that there appear to be glycosylation differences between E2a and E2b. Neither precursor labeled the C protein, indicating that C is not glycosylated and that the sugar labels were not metabolized into amino acids during the labeling period. [^{35}S]methionine-labeled virus was treated with neuraminidase, which removes terminal sialic acid residues. E2a and E2b migrated slightly faster (Fig. 1, lane 9) than the corresponding proteins from the untreated control (Fig. 1, lane 8), whereas the mobility of E1 was unchanged. The same results were obtained also if the virus was first disrupted with Triton X-100. Treatment of the virus with endoglycosidase H, an enzyme that cleaves high-mannose-type gly-

cans between the two core *N*-acetylglucosamine residues (31), had no effect on the mobilities of any of the proteins, suggesting that the glycans are all of the endoglycosidase H-resistant type.

Tryptic peptide analysis. To study the structural relationship between the four proteins, [^{35}S]methionine-labeled proteins were separated on a preparative SDS-polyacrylamide gel, detected by autoradiography (Fig. 2A), and eluted from the gel. The purity of the eluted proteins was checked by re-electrophoresis of a sample of each protein preparation (Fig. 2B, lanes 2 to 5). The proteins were then oxidized with performic acid and digested with trypsin. The resulting [^{35}S]methionine-labeled peptides were analyzed by reverse-phase HPLC and by two-dimensional separation on cellulose thin-layer plates.

The elution profiles obtained from a reverse-phase column (μBondapak) are shown in Fig. 3A through D. It is evident that the elution profiles of E2a and E2b are very similar, suggesting a close structural relationship, whereas E1 and C are different entities. Principally, the same results were obtained when two other column types (Spherisorb S5-phenyl and Vydac 201TP) were used (data not shown). The absolute retention times of individual peptides were different in each column, but, again, the elution profiles of E2a and E2b were nearly identical.

Peptide maps of E2a and E2b obtained on cellulose thin-layer plates are shown in Fig. 4A and B. The results confirm those obtained by HPLC: the maps from E2a and E2b are very similar, if not identical. The maps from E1 and C were different from each other and differed also from that from E2a (or E2b) (data not shown). In fact, the [^{35}S]methionine-containing tryptic peptides from E1 migrated very poorly on the cellulose thin-layer plate, causing only a smear around the origin. The same result was obtained after three individual trypsin digestions and separations. Probably, the [^{35}S]methionine-labeled tryptic peptides from E1 are too large (or hydrophobic) to be separated well on a cellulose thin layer. This is evident also from the HPLC result, where the tryptic [^{35}S]methionine-containing peptides eluted unusually late (main fragment in fractions 76 to 78, 40% CH_3CN) from the μBondapak column and even later from the two other columns used. This behavior is typical for hydrophobic peptides (6).

Separation of the viral nucleocapsid and envelope. ^{32}P-labeled RV and [^{3}H]uridine-labeled SFV, another togavirus (12, 26), were mixed and treated with Triton X-100. The disrupted viruses were then fractionated on a 15 to 30% sucrose gradient. A radioactive peak sedimenting to the middle of the gradient was obtained for both viruses (Fig. 5). Taking the sedimentation rate of the SFV nucleocapsid as 150S (29), the S value

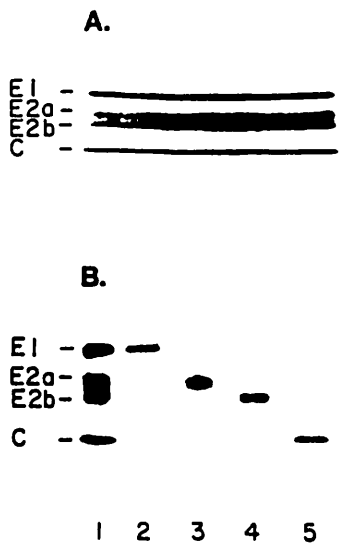

FIG. 2. Preparative SDS-gel electrophoresis of [^{35}S]methionine-labeled RV proteins. (A) About 4 × 10^6 cpm of purified virus was disrupted with SDS and applied to a 5 to 16% polyacrylamide gradient gel. After electrophoresis the gel was dried and autoradiographed for 15 h. (B) Samples from the eluted protein preparations were re-electrophoresed in a similar gel. Whole virus proteins were used as markers (lane 1).

for the RV nucleocapsid was calculated to be about 160 (20). The material left at the top of the gradient represented the envelope fraction. In a similar experiment, in which the virus was labeled with [^{35}S]methionine, the nucleocapsid peak (about 20% of the radioactivity) contained only the C protein (Fig. 1, lane 2), whereas the envelope (80%) contained glycoproteins E1, E2a, and E2b (Fig. 1, lane 3) as analyzed by SDS-gel electrophoresis.

Verification of E1, E2a, and E2b as externally located proteins. Purified unlabeled virions were labeled with [^{3}H]borohydride after treatment with neuraminidase and galactose oxidase. This procedure labels only externally located glycoproteins that after removal of the sialic acids contain terminal galactose or galactosamine residues (7). In particular, E2a (Fig. 1, lane 7), but also E2b and to a much lesser extent E1, were labeled, suggesting an external location. To verify that these proteins are indeed externally located, [^{35}S]methionine-labeled virus was treated with pronase, and the protease was removed by centrifugation on a sucrose density gradient. The treated particles still banded as a sharp peak, but at a reduced density. Analysis of the protein composition of these particles showed the presence of only C protein, whereas E1, E2a, and E2b had been completely removed (data not shown).

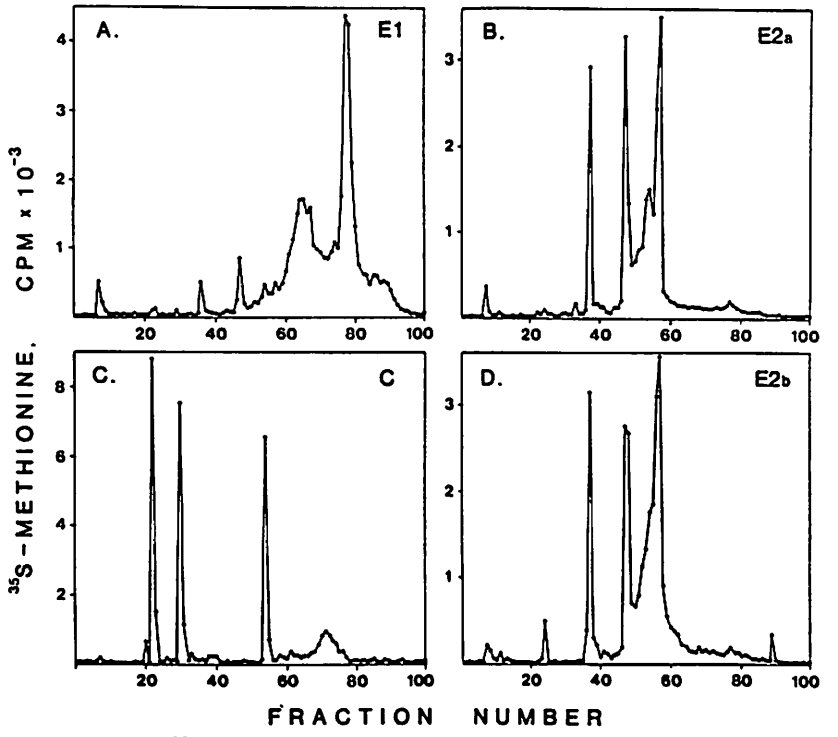

FIG. 3. Separation of the [^{35}S]methionine-labeled tryptic peptides from RV structural proteins by HPLC. Chromatography was on a μBondapak column (3.9 by 300 mm) in a linear gradient of acetonitrile (0 to 63% in 60 min) in 0.09% trifluoroacetic acid at a flow rate of 1.0 ml/min. Fractions of 0.5 ml were collected and assayed for radioactivity. Elution profiles of peptides from E1 (A), E2a (B), C (C), and E2b (D).

Solubilization of the envelope proteins. Treatment of enveloped RNA viruses with Triton X-100, followed by removal of the nucleocapsid, lipids, and detergent by sucrose gradient centrifugation, results in the formation of soluble viral spike complexes ("rosettes") (9). The complex formation presumably occurs due to the presence of a stretch of hydrophobic amino acids usually located at the C-terminal end of the glycoproteins. [^{35}S]methionine-labeled RV was disrupted with Triton X-100, and the nucleocapsid was removed by centrifugation as described above (Fig. 5). The envelope proteins were then complexed by centrifugation on another sucrose gradient. About 80% of the radioactivity sedimented as a rather homogeneous peak, whereas the rest remained at the top of the gradient (Fig. 6). The peak fraction contained E1, E2a, and E2b in about the same ratio (Fig. 1, lane 4) as found in the virions (lane 1) and total envelope fraction (lane 3). The material left at the top of the gradient also contained all three proteins and probably represents the monomeric uncomplexed forms of the spike proteins. Electron micrographs taken from the peak fraction after dialysis showed star-shaped particles (Fig. 6, inset), typical for envelope glycoprotein complexes (9, 14, 16, 27, 28).

Analysis of RV-specific intracellular proteins made in the presence and absence of tunicamycin and monensin. To study the synthesis of RV-specific proteins made in infected cells, an antiserum was prepared in rabbits against purified SDS-disrupted whole virus. The reactivity of this serum was checked by immunoblotting (34). The serum reacted with all four proteins. The reactivity with E1 and C was much stronger than with E2a and E2b (data not shown).

Tunicamycin is known to inhibit the addition of asparagine-linked glycans to proteins, resulting in the synthesis of unglycosylated proteins (30, 33). To study the effect of tunicamycin on the synthesis of RV glycoproteins, infected cells were pretreated for 3 h with tunicamycin and then pulse-labeled in the presence of the drug for 1 h. Untreated cells served as a control. After a 30-min chase, cell extracts were prepared, and the virus-specific proteins were immunoprecipitated with the antiserum. Polypeptides that comigrated with E1 and C were immunoprecipitated (Fig. 7, lane 2). In addition, a polypeptide migrating slightly faster than E2b (M_r, ca. 41,000) was also efficiently precipitated (Fig. 7, lane 2, arrow), whereas no polypeptide migrating at the position of E2a was seen. In the presence of tunicamycin (Fig. 7, lane 3), the

migration of E1 and the 41,000-dalton protein, which probably represents E2, was clearly altered. The unglycosylated forms of E1 and E2 proteins migrated with an M_r of about 53,000 and 30,000. The identity of the polypeptide that migrated slightly faster than E2b and was precipitated from tunicamycin-treated cells (Fig. 7, lane 3) was not established. The mobility of the RV-specific proteins was unaltered by the presence of monensin (Fig. 7, lane 4), a drug that inhibits the transport of glycoproteins from the Golgi complex to the plasma membrane (32).

DISCUSSION

A thorough reexamination of the structural proteins of RV was prompted by the confusing data published during the last 10 years. Since the original paper by Vaheri and Hovi (37), which indicated the presence of three structural proteins in RV, conflicting reports as to the number and size of the RV proteins have been published (2, 5, 11, 12, 18, 21, 36, 37, 39). We have here characterized the structural proteins of RV by a number of different methods and found four major proteins in highly purified virus preparations. By using the nomenclature adopted for togaviruses (13), these proteins were designated E1 (58,000 daltons), E2a (47,000 daltons), E2b (42,000 daltons), and C (33,000 daltons).

The unglycosylated C protein was associated with the RNA, whereas E1, E2a, and E2b were glycosylated and located externally on the viral envelope as shown by labeling with radioactive sugar precursors, fractionation of the nucleocapsid and the envelope, and external labeling with [^{3}H]borohydride after treatment with galactose oxidase. These proteins were also susceptible to pronase digestion. All three glycoproteins participated in the formation of soluble glycoprotein complexes after removal of the viral lipids and the detergent by sucrose gradient centrifugation, suggesting that they are integral membrane proteins (8). Similar complexes have been previously described for several viral glycoproteins (9, 14, 16, 27, 28). Recently, Trudel and Nadon (35) have described the formation of membrane protein complexes from RV. In that report, the protein composition of the complexes was not, however, determined.

Several authors have found at least three major polypeptides in RV with molecular

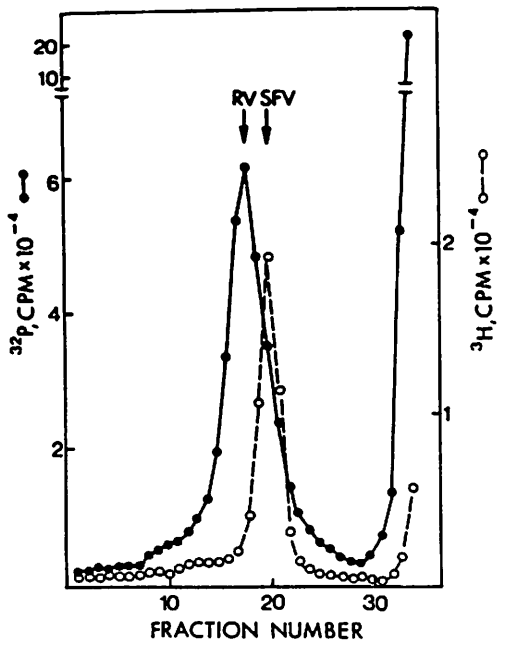

FIG. 5. Fractionation of RV nucleocapsid and envelope by sucrose gradient centrifugation. ^{32}P-labeled RV and [^{3}H]uridine-labeled SFV were treated with 2% Triton X-100 and layered on a 15 to 30% sucrose gradient. Centrifugation was for 4 h at 39,000 rpm in an SW41 rotor. Fractions of 0.3 ml were collected from the bottom and assayed for radioactivity.

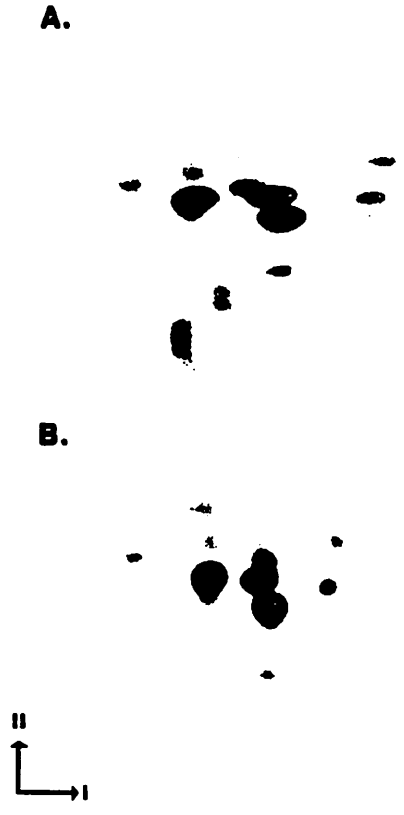

FIG. 4. Maps of [^{35}S]methionine-labeled tryptic peptides from RV structural proteins E2a (A) and E2b (B) after two-dimensional separation on cellulose thin-layer plates.

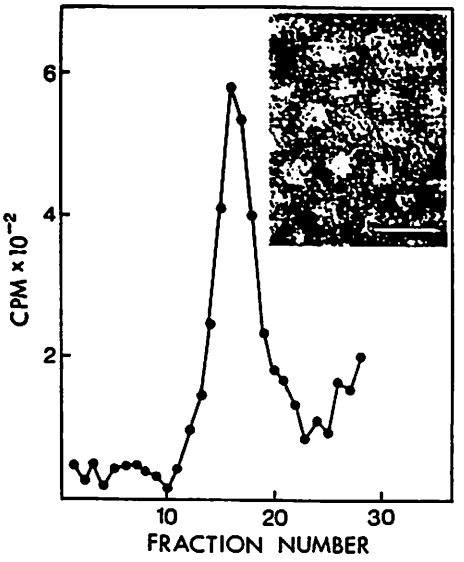

FIG. 6. Complex formation of RV envelope glycoprotein in a sucrose gradient. Triton X-100-disrupted ^{35}S-labeled RV was layered on a sucrose gradient (see the text) and centrifuged for 20 h at 39,000 rpm and 20°C in an SW41 rotor. Fractions of 0.4 ml were collected from the bottom and assayed for radioactivity. An electron micrograph of RV envelope glycoprotein complexes (fraction 16) negatively stained with potassium phosphotungstate is shown in the inset. Bar = 50 nm.

weights of 55,000 to 63,000, 42,000 to 50,000, and 30,000 to 35,000 (2, 11, 12, 21, 36, 37) that probably correspond to our E1, E2a, or E2b, and C proteins. Others have found major polypeptides with molecular weights of, e.g., 44,000, 41,000, 24,000, and 19,000 (39) or 63,000, 60,000, 32,000, and 30,000 (17). In addition, a number of minor polypeptides of various sizes have also been reported (2, 17). There appears to be a consensus that E1 and E2a, or E2b (using our nomenclature) are envelope-associated glycoproteins and that the C protein is associated with the RNA, forming the nucleocapsid (5, 26, 37).

The protein composition of RV is similar to that of the alphaviruses (10, 13), which have a capsid protein (M_r = 33,000) and two large glycoproteins (M_r = 51,000 and 52,000). In contrast, RV proteins clearly differ from those of the flaviviruses and several other non-arthropod-borne togaviruses (e.g., hog cholera, bovine diarrhea, lactic dehydrogenase, equine arteritis viruses) (5, 10). None of the above viruses appears to contain a protein which is present in two different forms. Thus, based solely on protein composition, the classification of RV in a separate *Rubivirus* genus within the Togaviridae (26) family seems to be justified.

The four proteins reported here are probably encoded by only three genes, since E2a and E2b were shown by tryptic peptide analysis to be very similar, if not identical, whereas E1 and C appeared to be unrelated to each other and to E2a or E2b. The reason for the difference in mobility in SDS-gels of E2a and E2b is not fully clear. They were both labeled with [^{3}H]glucosamine, whereas [^{3}H]mannose labeled predominantly E2b. These results suggest a difference in the glycan moiety of E2a and E2b. The mobilities of the proteins were unaffected by the treatment with endoglycosidase H, suggesting that their glycans were not of the high-mannose type (31). The external labeling with [^{3}H]borohydride of E2a after treatment with galactose oxidase does not exclude the presence of complex glycans in this protein. The mobility difference between E2a and E2b could thus be in the number of glycans. At present, differences in the glycan structures cannot, however, be excluded. The difference in labeling intensity of the three glycoproteins with [^{3}H]borohydride may relate to the accessibility of the glycans to the labeling procedure, as has been shown to be the case for the glycoproteins of SFV (19).

Analysis of RV-specific proteins by immuno-

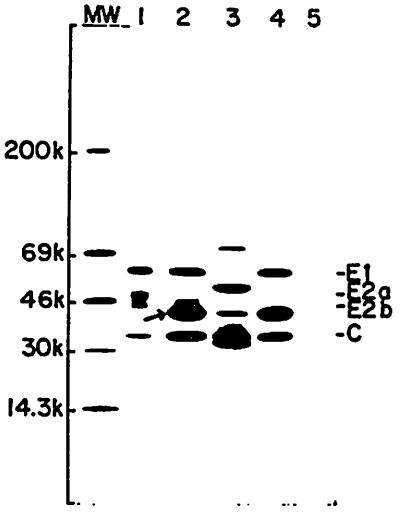

FIG. 7. Analysis of immunoprecipitated RV-specific intracellular proteins. RV-infected Vero cells labeled with [^{35}S]methionine for 1 h in the absence (lane 2) or presence of tunicamycin (lane 3) or monensin (lane 4). The proteins were immunoprecipitated with RV antiserum made in a rabbit and analyzed on a 5 to 16% SDS-gradient gel. Precipitation of infected cell extracts with preimmune serum was used as a control (lane 5). Lane 1, [^{35}S]methionine-labeled RV. The arrow in lane 2 indicates the position of the 41,000-dalton protein. Molecular weight markers in thousands are shown on the left.

precipitation of [^{35}S]methionine-labeled cytoplasmic extracts revealed only one major polypeptide migrating in the approximate position of E2b. This protein apparently contained asparagine-linked glycans, since it could not be detected in tunicamycin-treated infected cells. Instead, a new immunoprecipitable protein migrating slightly faster than the capsid protein was seen. Presumably, this protein represents the unglycosylated form of E2b or E2a or both. The fact that E2a was not seen in the cellular extract after a 1-h pulse and 30-min chase suggests that the posttranslational modifications resulting in the formation of E2a occur late during virus maturation. Amino-terminal and carboxy-terminal sequence analyses, as well as characterization of the glycan moieties, are needed to fully understand the differences between E2a and E2b. The analysis of [^{35}S]methionine-labeled tryptic peptides does not exclude the possibility of amino-terminal or carboxy-terminal differences between these two proteins, since a cleavage product from E2a could be devoid of methionine residues and therefore would remain undetected. Two glycoproteins with molecular weights of 47,000 and 45,000, probably corresponding to our E2a and E2b, have recently been reported by Ho-Terry and Cohen (12) to be present in RV. These proteins had different isoelectric points and also yielded tryptic peptides indistinguishable from each other.

Assuming that there are three genes coding for the structural proteins E1, E2a or E2b, and C and that the apparent molecular weights of the unglycosylated apoproteins are 53,000, 30,000, and 33,000, respectively (Fig. 7), the structural genes must have a coding capacity for about 116,000 daltons of protein. A subgenomic 24S RNA species has recently been detected in RV-infected cells (C. Oker-Blom, unpublished data), possibly corresponding to the 26S subgenomic mRNA of alphaviruses that encodes the 130,000-dalton precursor of the structural proteins (13). Thus, a 116,000-dalton precursor of the RV proteins, if such a precursor exists, could well be encoded by the 24S RNA species.

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STUDIES ON MEASLES VIRUS
II. PROPAGATION IN TWO ESTABLISHED SIMIAN RENAL CELL LINES AND DEVELOPMENT OF A PLAQUE ASSAY

By

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Introduction

Since the first isolation of measles virus in human and rhesus monkey kidney cells by Enders and Peebles[1], the laboratory strains of measles virus have been grown in various cells[2~17]. During our investigation of selecting cell system suitable for virus growth and for virus titration, it was found that measles virus could grow readily in MS-cell cultures (a continuous cell line of rhesus monkey kidney established by Dr. Tytell[18])[19], and in VERO-cells (a continuous cell line of green monkey kidney established by Dr. Yasumura[20]. In VERO-cells cytopathic changes appeared earlier than in FL-cells which have been employed commonly for measles virus titration, and the infected cells were maintained continuously in medium without serum for more than 7 days. We will propose that VERO-cell is one of the most excellent host cell for viral assays in studies on measles virus.

By other authors, it has been reported that measles virus show to form plaques on some cell monolayer cultures[21~25]. We found also that measles virus produced distinct plaque with clear center and sharp boundary.

This report describes our investigations on these host cells which we have employed for studies on variation of measles virus. They are results which we have made on the growth rate of the virus in these two cell systems and on the development of a plaque assay which permits more quantitative studies with the virus and cloning the virus.

Materials and Methods

1. Measles Virus

The Edmonston strain of measles virus was most extensively employed. The passage history of this strain until when it was received in our laboratory, was

described previously[19]. The original virus supplied was serially passaged in each FL- and MS- cells as stock virus.

The Sugiyama strain of measles virus[26] was used in some experiments. This strain was kindly supplied by Dr. Matsumoto in Institute for Infectious Diseases of Tokyo University. When it was received, it had been passaged in 6-monkey kidney cell cultures and in 8-FL cell cultures. In our laboratory, it was further serially passaged in VERO-cells as stock virus.

2. Cell Cultures

The growth medium and the maintenance medium of MS-, FL-, and HeLa-cells were prepared with composition as described previously[19]. The growth medium of VERO-cells was prepared with YLE. $CS_2PVP_{0.1}$—, YLE, 97.9%; Calf Serum, 2%; Polyvinylpyrrolidone, 0.1%; (at final concentration of bicarbonate, 0.11%) established by Dr. Yasumura[20]. The maintenance medium of VERO-cells infected with the virus was LE-medium (Earle's solution consisting 0.5% lacto-albumin hydrolysate without serum) which was changed once weekly as his report[20].

3. Titration of Infectivity

One-log dilutions of the sample to be assayed for virus content were made in LE-medium. One-fifths of one ml aliquot of each dilution was added to each of 4 or 10 roller tubes which contained cells of 3-5 day cell old and were removed nutrient fluid before inoculation. After they were placed for 1 hr. at 37°C, 1.5 ml of maintenance medium was added to each tube, and then were cultured at 37°C. When the typical cytopathic effect of measles was noted, the tube was recorded as positive. The observation on the tubes were made at intervals until the 14th day post-infection. The experiment was then terminated and the infective titer of the virus was calculated by the method of Reed and Muench.

4. Serum Neutralization Tests

The all sera tested were inactivated by heating for 30 min. at 56°C. Serial 2-fold dilutions of the serum were then made in LE-medium. To each tube of diluted serum was added same volume of a solution containing approximate 600 TCD_{50}/0.2 ml of measles virus in LE-medium. The tubes were stoppered, shaken and then incubated for 1 hr. at 4°C. After incubation, 0.2 ml of aliquots (300 TCD_{50} of virus) from each tube was added to each of 3 roller tubes in same manner on infectivity titration. The tubes were examined microscopically 24 hrs. later for evidence of serum toxicity. The final reading for virus was made on the 14th day post-infection, and the serum neutralizing titer was recorded as the highest dilution of serum which protected 50% of the cultures.

5. Plaque Assays

The plaque methods will be described which have given consistent results in our investigation. MS- and VERO-cells were grown in rubber-stoppered small square bottles (2-Oz). The bottle was seeded with approximately 9×10^5 cells in

6 ml of growth medium described above. They were incubated for 2 to 3 days at 37°C until when complete monolayers were established. The day before virus inoculation, the medium was changed with fresh one. On the day of virus inoculation, after removal of the medium each of monolayers was washed once with phosphate buffered salt solution (PBS), the bottle was inoculated with 0.2 ml of appropriate dilutions of the virus in PBS. The bottles were then placed at 37°C after shaking to assure a uniform distribution of virus. After incubation which would be described below the suitable time on each cell monolayer, a solid overlay was added. The overlay was prepared by ingredients as shown in Table 1, which was given best results in our investigations. Equal parts of the agar suspension (at 42°C) and the nutrient fluid (at 37°C) were mixed immediately before pouring the overlay. Four ml of the mixture was poured on each VERO-cell bottle, but 3 ml in case of MS-cells. After the overlay was solidified, the bottles were turned over and placed at 37°C. In case of using MS-cells monolayer, a second overlay of same composition was added on the fifth day of incubation, and then 0.2 ml. of 0.1% neutral red in PBS was added on the sixth day of incubation. Subsequently the bottles were incubated in the dark.

Table 1. *Composion of Overlay Medium in Each Cell Type*

Ingredients		MS-Cell		VERO-Cell
(A).				
1. YLE (3×concentrated without phenol red and $NaHCO_3$)	—	60.0 ml	—	60.0 ml
2. Calf Serum	—	3.6	—	0
3. 7.5% $NaHCO_3$	—	5.4	—	5.4
4. 0.1% Neutral Red	—	0	—	3.6
5. Antibiotics (Penicillin, 2×10^4 U; Streptomycin, $2\times10^4\gamma$)	—	1.8	—	1.8
6. Distilled water	—	19.2	—	19.2
Total	—	90.0 ml	—	90.0 ml
(B).				
1. Noble Agar (Difco)	—	2.7 gm	—	2.7 gm
2. Distilled Water	—	90.0 ml	—	90.0 ml
Total (3% agar)	—	90.0 ml	—	90.0 ml

Agar suspension, to sterilize at 15 pounds for 30 min; allow to cool to 42°C before use.

6. Fixation and Staining

The cells grown on the slide slip and cell sheet shown to form plaques overlaid with nutrient agar were most extensively fixed by FAS (Neutral formalin, 12%; Acetic acid, 1%; PBS, 87%) for hematoxylineosin (H-E) stain.

In case of staining of cell sheet appeared plaques, FAS was poured in the plaque bottle and then after fixation the agar layer was gently stripped from the cell sheet attached on glass surface. The cell sheet washed off the excess FAS,

was stained by collodion membrane technic[1]. For H-E staining the routine technique was employed.

Results

1. *Morphological Observations on the MS- and VERO-cells Infected with Measles Virus*

The cytopathic changes induced by the Edmonston strain or the Sugiyama strain in MS- and VERO-cells were much the same as those described by Enders and Peeble[1]. The effect could be described as "giant multinuclear cell" and "syncytial formation" or "syncytial masses". In fixed and stained preparations, irregular inclusion bodies of strongly eosinophilic amorphous material surrounded by clear zone were observed in the cytoplasm and eosinophilic intranuclear bodies with hallos were often seen at late stage (Fig. 5~8). In both MS- and VERO-cells cytopathic effect was easily observed in fresh preparations inoculated with laboratory measles virus strains. Especially, though observation was done later than 10 days postinfection, cytopathic effect in VERO-cells was seen most clearly than those in FL-, MS- and HeLa-cells because VERO-cells were maintained best until 14th day postinfection.

By neutralization tests, it was found that these cytopathic changes appeared in both cells were inhibited completely by specific measles antisera diluted to contain 20 units of neutralizing antibody and also by human measles convalescence serum, but antisera of each normal FL-cells and MS-cells prepared in the same manner did not possess inhibition of the cytopathic changes. These measles antisera were obtained to inoculate repeatedly with each Edmonston strain of FL-passaged virus line and MS-passaged virus line into rabbits and guinea-pigs.

These results indicate that measles virus is not varied antigenically by passaging serially in FL-, MS-, or VERO-cells.

2. *Comparison of Susceptibility of Three Different Cell Cultures to the Various Cell-passaged Virus Lines and to the Two Virus Strains*

For the purpose of appreciating susceptibility of MS- and VERO-cells to measles virus, the following experiments were carried out. As the control cell in the experiments it was employed FL-cell which had been used commonly for assays of measles virus.

Infectivity titrations were repeatedly carried out at the same time using the same conditioned cell tubes (cell old and concentration of cells) of each type of cells. The tubes were inoculated with the same aliquot of each dilution of virus samples, and were recorded daily number of tubes appeared cytopathic effect in each group of cell types.

The curves plotted titers which were detected daily in each group of cell type were compared upon each same test.

This test was carried out on the following virus lines; the two stock virus

lines (FL-passaged virus and MS-passaged virus) of the Edmonston strain, the line passaged serially in VERO-cells from these stock viruses, and also the VERO-passaged virus of the Sugiyama strain.

The ascending curves of titers of the above virus lines using MS-cells exhibited to be not different from those in FL-cells. However, maximum titer in

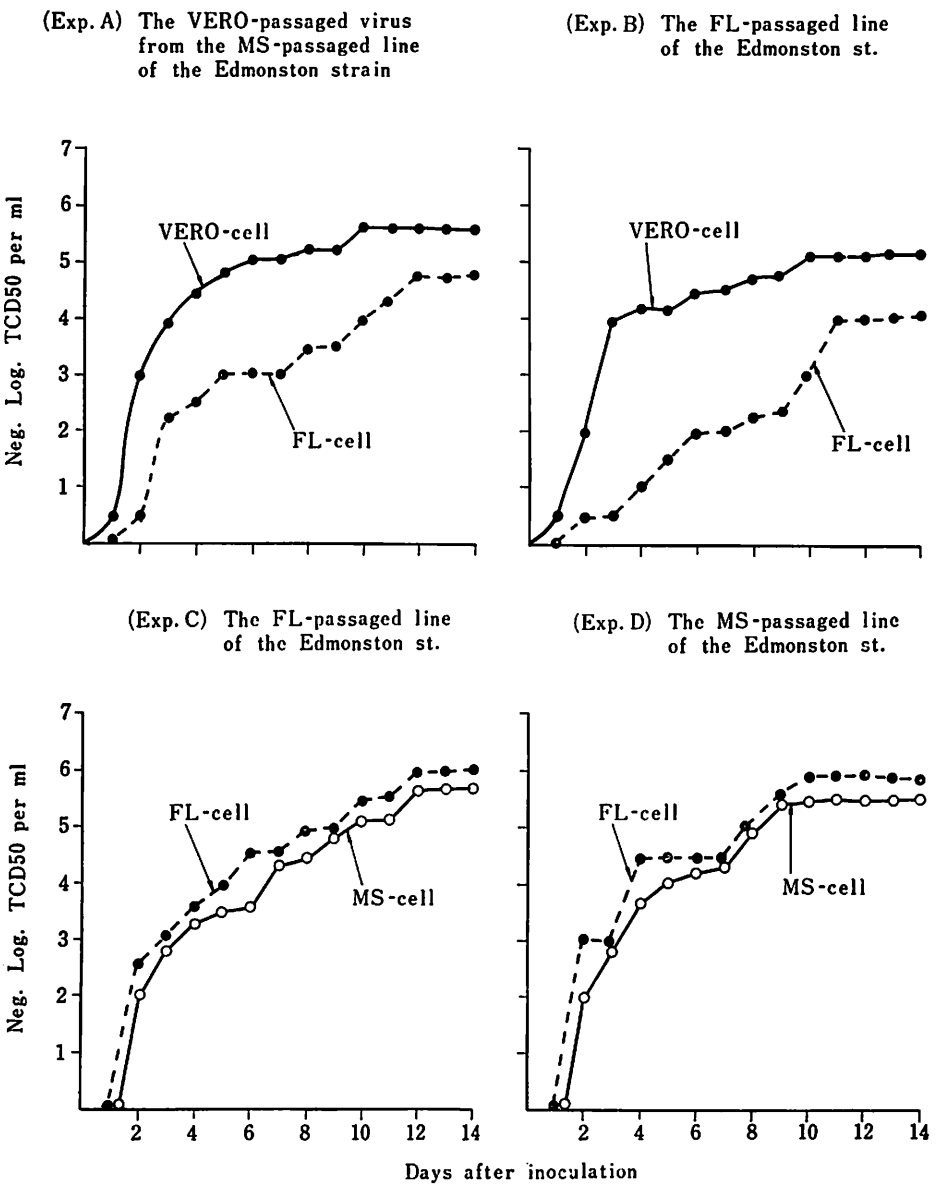


Fig. 1. *Comparative Titration of Various Virus Lines Using Tubes Contained Each of Three Cell Types*

VERO-cells was half to one log units higher and detected 2 to 3 days earlier than in the two other cells. A cytopathic effect which was firstly observed in VERO-cells, was also detected 1 to 2 days earlier in same dilution of virus than in the other cells. One of our experiments was shown in Fig. 1.

The maximum titers per ml obtained from various inoculum-size (0.1, 0.2 and 0.5 ml) showed nearly equal in each of cell type.

3. Attempts to Isolate Measles Virus from Patients

During outbreak of measles Tokyo in 1962, isolations of measles virus from 8 heparinized bloods and 7 throat swabs obtained from 8 patients as early as possible after a clinical diagnosis of measles was established (Fever stage before rash or within 24 hrs. after rash), were attempted using MS-cells. Procedures for isolation were employed techniques of EDTA treatment[14]. The materials were serially passaged at interval of 14-21 days until 2nd to 5th generation, but the results were all negative. Previous attempts to isolate virus from the collected 32 specimens of 31 patients during outbreak of 1961 in Tokyo using FL-cells which were employed successfully by Frankel, et al.[10] and Toyoshima, et al.[14] (in our experiments passaged materials were treated 3 times with freez-thawing), gave negative results.

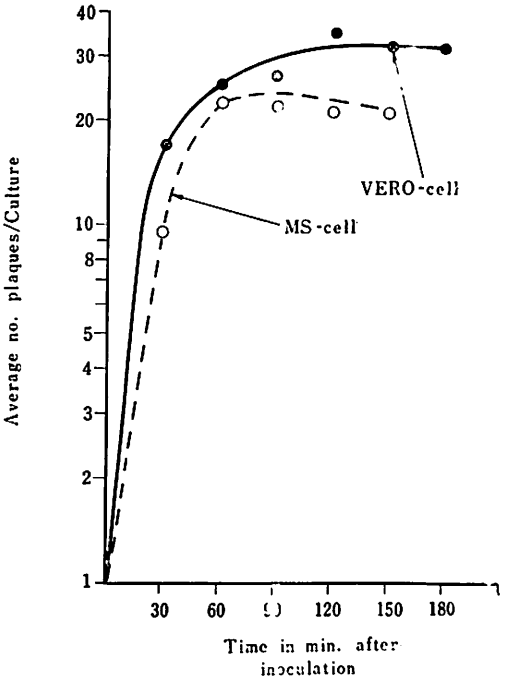


Fig. 2. *Adsorption of Measles Virus to Monolayer Cultures of MS- and VERO-cells at 37°C*

4. Development of Plaque Assays Using Two Cell Lines

It was found that measles virus produced easily distinct plaques with clear centers and sharp boundaries on both cell monolayer with nutrient agar described above.

They ranged in diameter between 0.5 and 1 mm at the time of maximum plaque count on MS-cell monolayer (Fig. 9), and on the other VERO-cells they showed 0.5~4 mm in diameter at the optimum count time in one bottle (Fig. 10). These plaques appeared microscopically to consist mainly of syncytial giant cells or syncytial masses in unstained and stained preparations (Fig. 11).

The adsorption rate of measles virus to both cell monolayer cultures washed with PBS was examinated to inoculate with a dilution of the virus suspension estimated to contain about 20~30 plaque forming units (PFU), and then to incubate at 37°C. At a definite interval of time after inoculation of the virus, groups of 4 cultures were removed from the incubator. They were washed immediately 3 times with cold PBS before overlaying with nutrient agar. Maximum adsorption occurred at 1 hr. on MS-cells and at 2 hrs. on VERO-cells, as shown in Fig. 2.

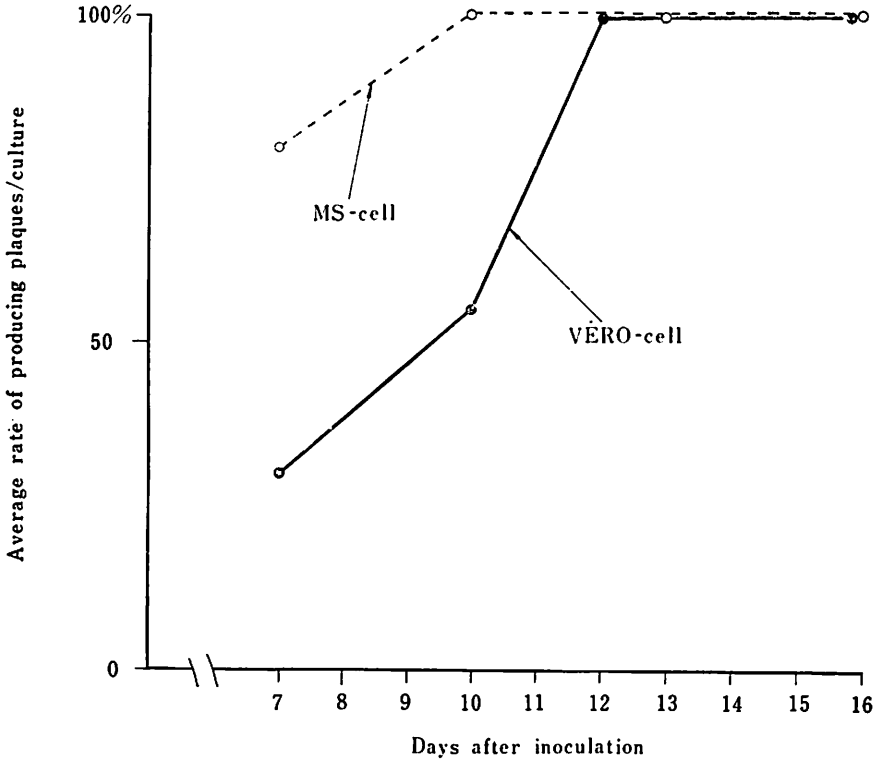
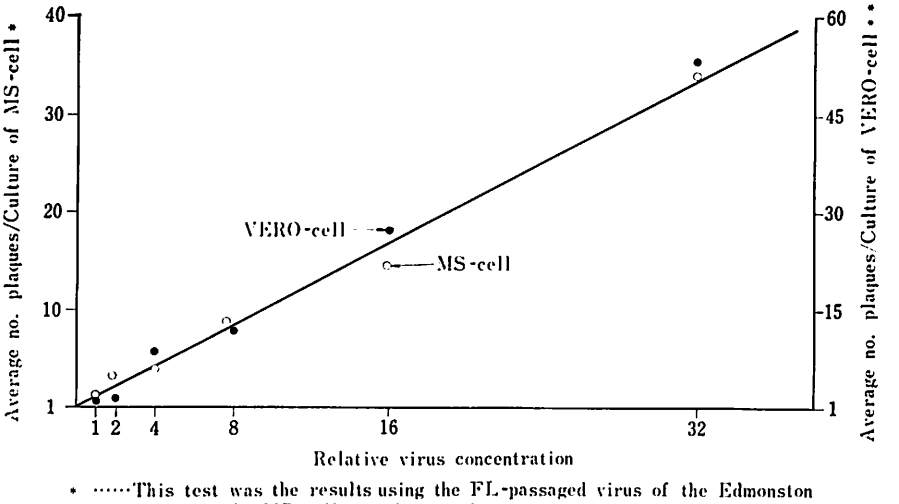


Fig. 3. *Incubation Time Required for Maximum Plaque Count at 37°C*

Incubation time required for maximum plaque count at 37°C on the both cells was determined by counting plaques at a definite interval of day in each of bottles used, for the purpose of determining the adsorption time. It would appear from the data of Fig. 3 that 10 days were required after infection for the appearance of all the plaques in MS-cell cultures, and in the other VERO-cells 12 days were required. (Fig. 3)

The relationship between plaque count and virus concentration was tested over a range of 6 serial 2-fold dilutions. Six replicated platings were made at each dilution and the average number of plaques per dilution in each of both cells was plotted as illustrated in Fig. 4. Each curve in the both cells is linear with a slope close to 1 suggesting that plaque is initiated by a single virus particle.



* ……This test was the results using the FL-passaged virus of the Edmonston strain on the MS-cell monolayer cultures.

* * ……The plaque assay using VERO-cell was carried out by inoculation of the MS-passaged virus line of the same strain.

Fig. 4. *Relationship between Plaque Counts and Relative Measles Virus Concentration*

To test the specificity of plaque formation by measles virus used, the plaque reduction tests were carried out using paired sera obtained from measles patient and specific measles rabbit anti-serum prepared to inoculate repeatedly with the FL-passaged virus line of the Edmonston strain, and as control serum, using the rabbit serum against normal FL-cells prepared in same manner.

Serial 2-fold dilutions of each serum were mixed with equal volume of the virus estimated to contain 50~100 PFU per inoculum and incubated for 1 hr. at 4°C. The mixtures were then assayed for plaque formation as described above. The results indicated that the human convalescence serum and rabbit antiserum against measles virus inhibited to form plaque, but control sera did not as shown

Table 2. *Neutralization of Measles Virus by Plaque Method*

Groups	Serum Dilution	Avg. PFU per One Culture of MS-cells
1. Control in absence of serum		49
2. Immune rabbit serum of FL-passaged virus of the Edmonston strain (# RS-14-108)	1: 16	0
	1: 64	0
	1: 256	3
	1:1024	40
3. Immune rabbit serum of normal FL-cells (# RS-19-108)	1: 4	45
4. Paired sera of measles patient (case no.1)		
a) Convalescense serum (# HS-4)	1: 8	0
	1: 16	8.5
	1: 64	16.5
	1: 256	21.5
b) Acute serum (# HS-1)	1: 4	33

This test was carried out at the same time by challenge of the MS-passaged virus of the Edmonston strain.

The results of other test using VERO-cell monolayer cultures exhibited nearly same.

in Table 2.

PFU obtained using each cell type exhibited nearly equal titer to that was estimated by roller tube method using the same cell type.

Discussion

It was found that laboratory measles virus grew easily in both MS- and VERO-cells as FL-cells which had been commonly used for viral assays, and antigenicity of the virus did not alter by serial passage in each cell type culture. By our experiments, we will propose that VERO-cells established by Dr. Yasumura is one of the most excellent host cell for viral assays in studies on measles virus.

For isolation of measles virus from human materials, MS-cells were unsuitable system, but whether VERO-cell is suitable such as primary human and simian tissue cultures, we will attempt to isolate the virus using this system at next chance of measles outbreak.

The observations that measles virus produced a cytopathic effect upon MS- and VERO-cells had led to the development of a plaque assay using these cell monolayer cultures.

The plaques were easily observed on these two cell systems. They were maintained sharply longer in time than on FL-cells (unreported data). In our experiences for 19~21 days after inoculation at 37°C, cell sheet of MS- or VERO-

cells was still in good state and the sharp plaques were observed.

On the monolayers of FL-cells for plaque assay, we often observed some bottles already contained dead cells at the 10th day after overlaying with nutrient agar.

The specificity of plaque formation by measles virus was established by plaque reduction tests using immune sera of measles virus. Furthermore, the virus clones from plaques were antigenically same. The small and large plaques appeared on VERO-cells were antigenically same[27]. However, whether they are variants of the measles virus, we are investigating at present.

The sensitivity of the plaque assays developed by us, was found to be nearly equal in each cell type to that of titration using roller tubes.

The plaque assay using MS- and VERO-cells should be applicable, therefore, to more meaningful quantitative studies of measles virus or of virus cloning.

Summary

1. It was found that laboratory measles virus produced easily typical cytopathic effect in MS- and VERO-cells. The VERO-cell was one of the most excellent system for viral assaying in studies of measles virus.

   Using MS-cells, isolation of measles virus from human materials was unsuccessful.
2. The procedures of the plaque assays using MS- and VERO-cells for measles virus infectivity have been described.

   The specificity of the assays was confirmed by plaque reduction tests and further, by identification of the virus clones from plaques.

Acknowledgment

We are grateful for the supply of patient materials by Dr. Toranosuke Kikuchi of Department of Pediatrics in Tokyo Hiroo Hospital, and by Dr. Hayami Arima and Dr. Tatsuo Ashidate of Department of Pediatrics in Tokyo Toshima Hospital. The able assistance of Mr. Mitsuo Naoi is gratefully acknowledged.

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The Cytopathic Changes appeared in the Three Different Cell Types by Infection of Measles Virus.

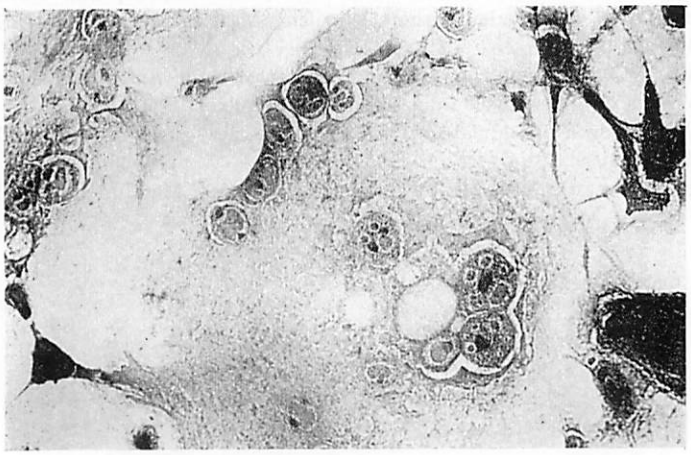

Fig. 5. *Syncytial formation containing both intranuclear and cytoplasmic inclusion bodies in the FL-cell culture infected with the FL-passaged virus of Edmonston strain. On the 20th day postinfection. H-E stain. ×200.*

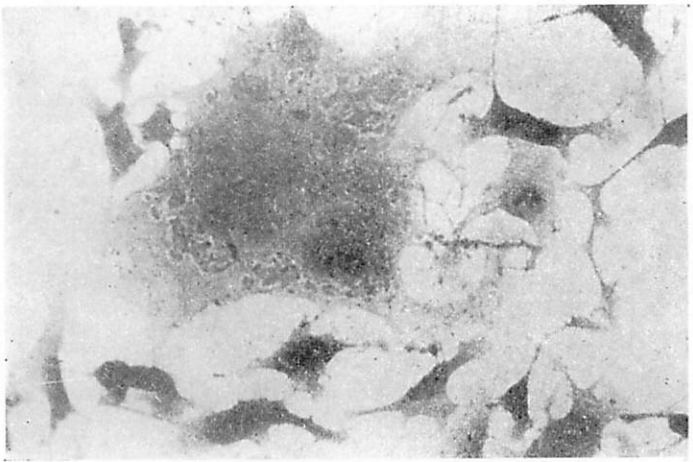

Fig. 6. *Syncytial giant cell containing both intranuclear and cytoplasmic inclusion bodies in the MS-cell culture infected with the virus described in Fig. 5. On the 10th day postinfection. H-E stain. ×150.*

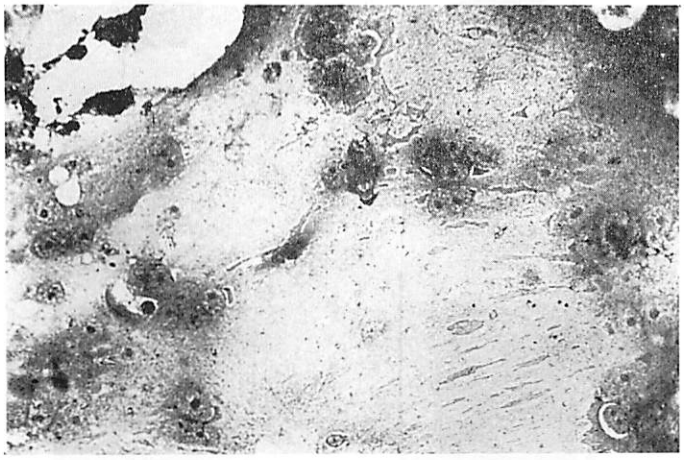

Fig. 7. *Syncytial masses containing cytoplasmic inclusion bodies in the VERO-cell culture on the 7th day after inoculation of the MS-passaged virus of Edmonston strain. H-E stain. ×100.*

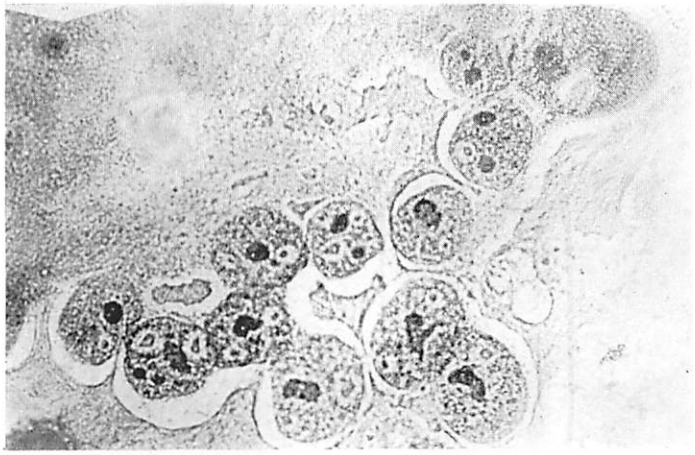

Fig. 8. *Syncytial formation containing both intranuclear and cytoplasmic inclusion bodies in the VERO-cell culture on the 12th day after inocualtion of the virus described in Fig. 7. H-E stain. ×600.*

Measles Plaques on the MS- and VERO-cell Monolayers

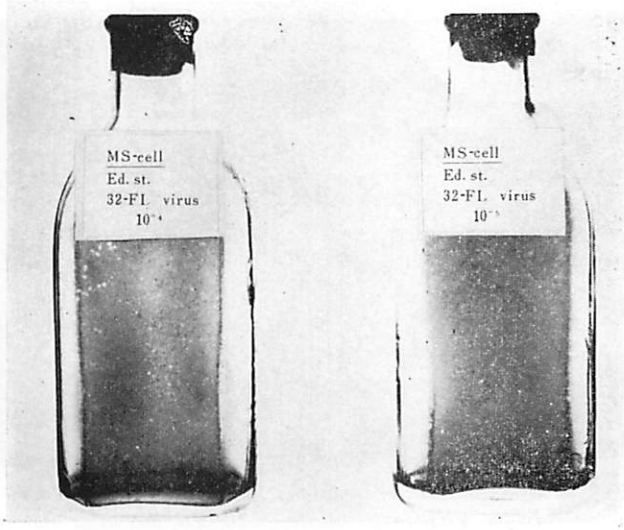

Fig. 9.
(On the 10th day after infection)

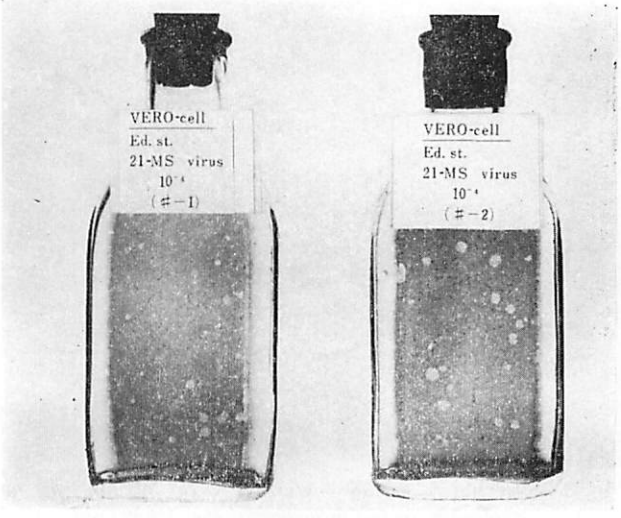

Fig. 10.
(On the 10th day after infection) *(On the 14th day after infection)*

Magnification of Measles Plaque on the VERO-cell Monolayer

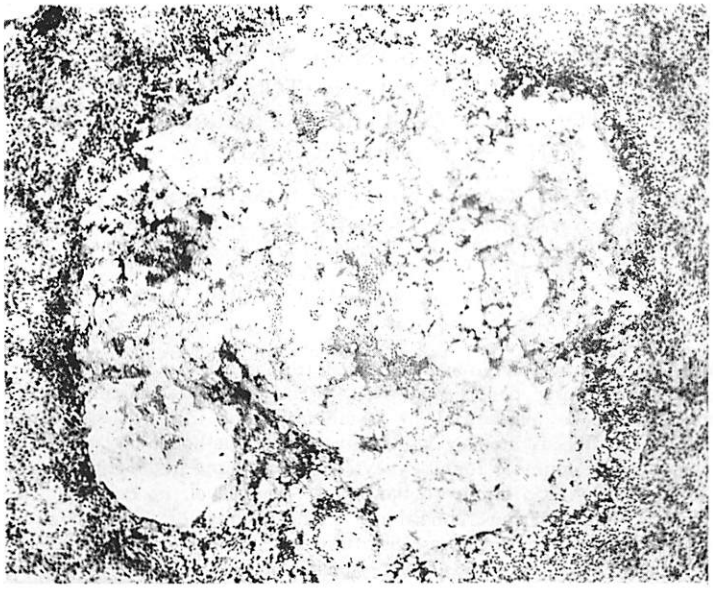

Fig. 11.
H-E stain. ×60.

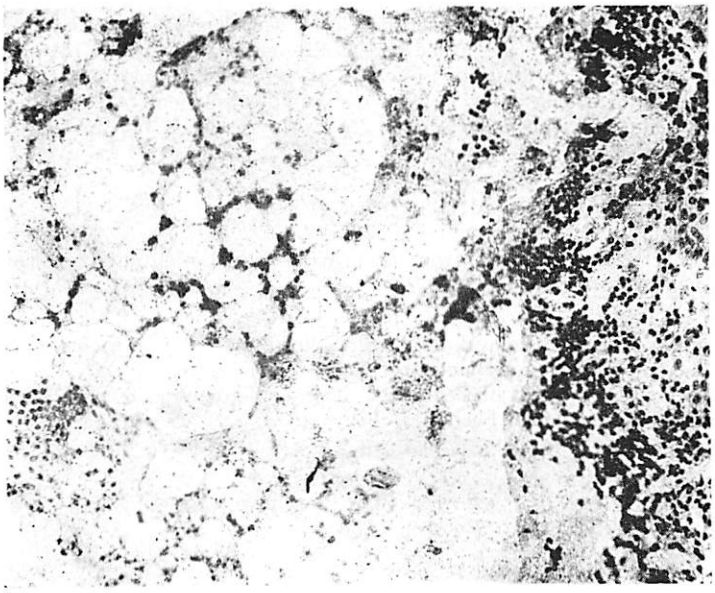

Fig. 12.
H-E stain. ×150.

Japan. J. Med. Sci. Biol., 25, 321-333, 1972

PROPERTIES OF A CYTOPATHIC AGENT ISOLATED FROM A PATIENT WITH SUBACUTE SCLEROSING PANENCEPHALITIS IN JAPAN

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SUMMARY: A cytopathic agent isolated from the brain biopsy of a patient with subacute sclerosing panencephalitis (SSPE) was investigated. The agent could most easily be maintained by cocultivation of infected cells with fresh Vero cells (cercopithecus monkey kidney line). Cytopathogenicity consisting of macroscopically recognizable round syncytia (plaques) appeared on the monolayer under liquid overlay. So far up to the 60th passage level, no cell-free virus has been obtained under various conditions. Antigenicity of measles virus was demonstrated by the immunofluorescence tests and by the plaque reduction tests using specific measles virus antiserum. In addition, electron microscopic findings of paramyxovirus nucleocapsids in the infected cells, hemadsorption of cercopithecus monkey erythrocytes and propagation characteristics in the presence of actinomycin D and of 5-bromodeoxyuridine were consistent with those of measles virus. That the nucleocapsids were abundant in the nuclei and much fewer in the cytoplasm is compatible with the data on SSPE viruses reported by others. Adult guinea pigs, mice and hamsters developed neurological symptoms by intracerebral inoculations of the agent and deaths ensued in most of the animals. Freezing storage with dimethyl sulfoxide at −70C was able to preserve the infected cells for a long period just as with uninfected Vero cells. This may suggest a temperate association of the agent with host cells and nonrecovery of cell-free virus.

INTRODUCTION

Isolation of cytopathic agents from brain biopsies of patients with subacute sclerosing panencephalitis (SSPE) was successful in several cases (Baublis and Payne, 1968;

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Katz, Oyanagi and Koprowski, 1969; Chen et al., 1969; Horta-Barbosa, Fuccillo and Sever, 1969; Kettyls et al., 1970) and viruses identical to or closely related to measles virus were recovered from cell culture systems by methods such as cell fusion and cocultivation (Horta-Barbosa et al., 1969; Chen et al., 1969; Payne, Baublis and Itabashi, 1969; Barbanti-Brodano et al., 1970; Kettyls et al., 1970). For the first time in Japan, Sato et al. (1972) isolated a cytopathic agent from a patient with SSPE. But this particular agent was unseparable from host cells through 60 passages performed to date. The strain was established by cocultivation of the brain cells from the patient's biopsy specimen with Vero or HeLa cells and subpassages of the cocultures with fresh Vero or HeLa cells. The activity of the agent was recognized and measured by appearance of syncytial plaques on the cocultivated monolayer. Antigens of measles virus were demonstrated by the immunofluorescence technique in the cells of the syncytia. Other findings supporting the association with measles virus were also obtained. The main purposes of the present paper are to describe characteristic features of the agent, especially regarding to nonrecovery of cell-free virus, its pathogenicity to experimental animals and techniques for maintenance of the agent by cell passages and by freezing storage.

MATERIALS AND METHODS

SSPE agent: The agent (Niigata/1 strain) for the present study was isolated from the brain biopsy specimen of an 11 year-old girl with typical SSPE symptoms. The strain wes established by cocultivation with Vero or HeLa cells. The Vero line was exclusively investigated at the 3rd to 60th passage levels.

Maintenance of the agent: Maintenance of the agent was made by subpassages of the infected cells and the freezing storage of them with 10 % dimethyl sulfoxide (DMSO). The former was performed by mixing the trypsinized SSPE-infected cells with fresh Vero cells and incubation at 36 C for several days until appropriate time for the subpassage. Five to thirty times in number of Vero cells were mixed with the SSPE-infected cells. The ratio became larger for later passages. For the freezing storage, our routine method for Vero cells was adopted. About five million per ml-cell suspension in the growth medium containing 10 % DMSO was distributed into ampoules in 1.0 ml amounts and kept frozen at −70 C.

Plaque counting: Biological activity of the SSPE agent was measured by the plaque counting. After an appropriate length of incubation, the culture vessels (4-oz prescription bottle) were decanted and stained with crystal violet (0.1 % in 20 % ethanol) after fixation with ethanol. Plaques were counted macroscopically. The linearity between the plaque counts and the number of the SSPE culture cells used for the cocultivation (Fig. 1) supported reliability of the method. The syncytial plaques appeared also under the agar overlay applied to the monolayer one day after the start of cocultivation. Agreement of the results under the liquid medium with those under the agar overlay further confirmed adequacy of the technique. However, under the liquid overlay, from around 5th day on, secondary plaques of smaller size often appeared and made the counting difficult and the agreement with the counts under the agar did not hold. Therefore, the routine counting was made on 3 to 5 days after incubation of the coculture.

Viruses used for the control tests: Measles virus (Edmonston strain) and herpes

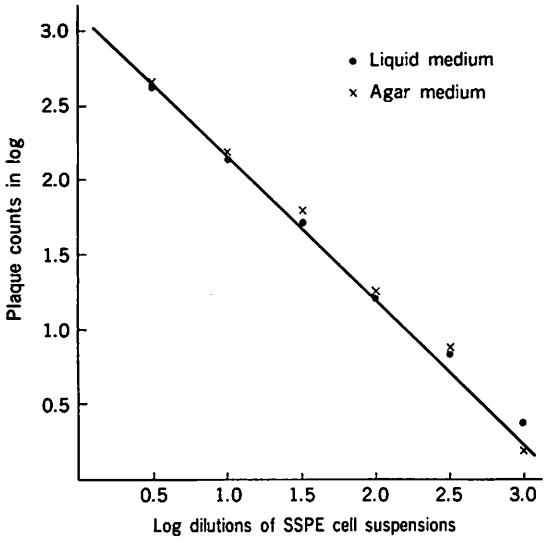

Fig. 1. Linear relationship between plaque counts and the dilutions of SSPE cell suspension used for cocultivation. Plaque counts were made after 3 days' cocultivation under liquid medium. They coincided with counts of plaques formed under agar overlay.

simplex virus (HF strain) were received from NIH of Japan and used after two passages in Vero cell cultures. Type 1 poliovirus (Sabin's vaccine strain) was provided by Japan Poliomyelitis Research Institute and used after two passages in primary cercopithecus monkey kidney cell cultures.

Antisera: All antisera used in the present work were prepared in Toshiba Institute of Biological Science. Rabbits were immunized with various viruses listed below. Specificities of the antisera were confirmed by cross neutralization tests or hemagglutination-inhibition tests. Measles (Edmonston), Rinderpest (LA), Canine distemper (Lederle), Mumps (Enders), Parainfluenza type 4 (M-25), RS (Long), Herpes simplex (HF), SV40 (VA-777): These strains were received from NIH of Japan. Parainfluenza types 1 through 3 (HA-2, CA, HA-1) viruses were given by Dr. R. M. Chanock.

Cell line and animal hosts: Vero cell line maintained for several years in Toshiba Institute of Biological Science was used throughout the present studies. The growth medium consisted of Earle BSS containing yeast extract (0.1 %), polyvinylpyrrolidone (0.1 %), bovine serum (2 %) and $NaHCO_3$ (0.19 %). The maintenance medium was Medium 199 with bovine serum albumin (0.1 %) and $NaHCO_3$ (0.225 %). As the antibiotics, streptomycin (100 μg/ml) and Kanamycin (100 μg/ml) were added to each medium. For the cocultivation of SSPE cells and Vero cells, the above growth medium was used. Experimental animals including mice, Syrian hamsters and guinea pigs were purchased from the local animal dealers.

Chemical reagents: The following reagents were used: Actinomycin D (Nippon Merck-Banyu Co., Japan); 5-bromo-2′-deoxyuridine (Nutritional Biochem. Co., U.S.A.); Thymidine (Sigma Chem. Co., U.S.A.); Dimethyl sulfoxide (Fuji Kasei Co., Osaka, Japan).

RESULTS

Cytopathogenicity of the Agent

Cytopathogenicity by the agent consisted of multinucleated syncytia. They became macroscopically recognizable around 10 hr after the start of cocultivation with Vero cells and reached 2 to 5 mm in diameter on the fifth day. However, destruction of the monolayer as a whole was not progressive. Enlargement of individual syncytia seemed to stop at certain limited sizes and they gave appearances of round clearly-bordered plaques, especially in the cultures that were kept undisturbed during the incubation. Figure 2 shows two adjacent plaques and a zone of normal cells between them. From around 5th day of incubation on, smaller secondary plaques[3] tended to appear. Eosinophilic inclusion bodies morphologically indistinguishable from those of measles virus were demonstrated by the hematoxylin and eosin staining in the cytoplasm and nuclei of the syncytia (Fig. 3). Hemadsorption of cercopithecus monkey

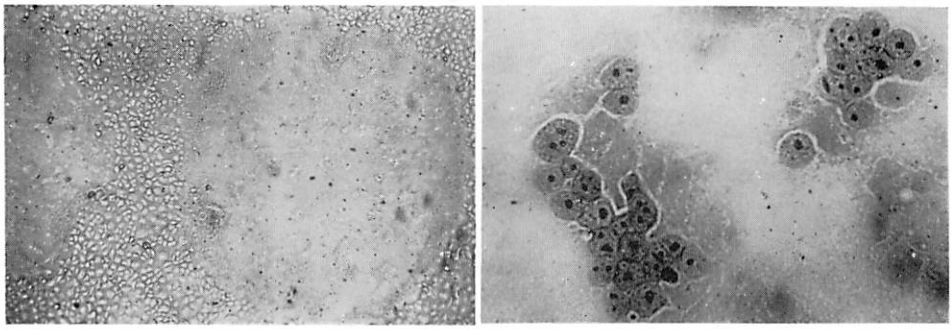

Fig. 2. Two adjacent syncytia in SSPE-Vero cell coculture.

Fig. 3. Hematoxylin-eosin stained syncytium showing cytoplasmic and intranuclear inclusion bodies.

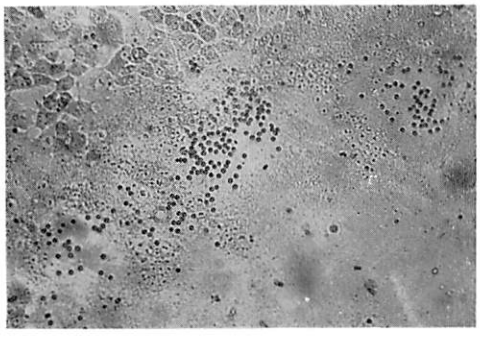

Fig. 4. Hemadsorption of cercopithecus monkey erythrocytes on the syncytium (8th passage).

3) They were interpreted by us as secondary ones originated from detached SSPE cells, because the floating cells in the coculture were shown to produce plaques on fresh Vero cell monolayer and attempted clonings of small plaque variants were unsuccessful.

erythrocytes was positive, but it was usually weak (Fig. 4) and did not become positive before the 8th passage. No hemadsorption of guinea pig erythrocytes was observed.

Plaque Reduction Tests

Typical results of the tests are shown in Table I. In these experiments, antisera against various viruses in their potent concentrations were added to the growth medium for cocultivation when the SSPE cells were suspended in it with fresh Vero cells. After 2 days of the cocultivation, the bottle cultures were emptied and plaque counts were made on the crystal violet-stained monolayers. Figure 5 shows the plaque reduction by the measles antiserum. From Table I, it is clear that the plaque formation was inhibited in the presence of measles antiserum. One of the rinderpest antisera

Fig. 5. Reduction of plaque counts by measles antiserum. Left to right; with 1:20 and 1:200 dilutions of antiserum and without antiserum.

TABLE I

Plaque reduction tests with antisera against various viruses

Experiment No.	Rabbit antiserum	Plaque counts	Percent reduction
1	None	196	—
	SV40	185	6
	Herpes simplex	195	1
2	None	40	—
	Measles	5	88
	Rinderpest	8	80
	Canine distemper	39	3
	HA-2	42	0
	CA	42	0
	HA-1	55	0
	M-25	54	0
	Mumps	45	0
	RS	46	0

SSPE cells were tested at 12th and 50th passage levels in experiments 1 and 2, respectively.

exhibited positive reduction, but no other heterologous antisera gave positive results. The plaque reduction was not consistent and became less pronounced when the counts were made later than 3 days after the start of cocultivation. The plaque reduction by measles antiserum was demonstrated repeatedly with the SSPE cells of different passage levels.

Immunofluorescence Studies

The acetone-fixed monolayers on coverslips were stained with monospecific FITC (Fluorescein isothiocyanate)-conjugated rabbit gamma globulins. They were prepared in our laboratory following the methods described by Kawamura (1969). The gamma globulins were fractionated from highly specific rabbit antisera as determined by the cross neutralization or hemagglutination-inhibition tests. After the labeling, the specificities were again confirmed by the cross staining test. As shown in Fig. 6, specific immunofluorescence was observed with the labeled measles virus antibody. Other antibodies against parainfluenza (types 1 through 4), mumps, RS and herpes simplex viruses gave negative results. The immunofluorescence was confined only to the syncytia and tended to be weaker than that of measles virus-infected cells. It was observed in cytoplasm and scarcely seen in nuclear positions. Immunofluorescent syncytia became few when the period of cocultivation exceeded 2 days.

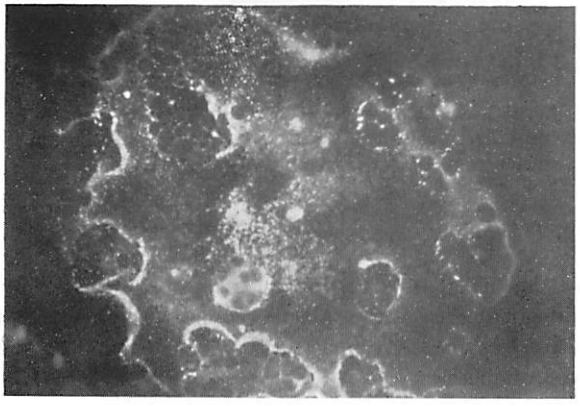

Fig. 6. SSPE syncytium stained with FITC-labeled anti-measles virus rabbit serum globulin.

Electron Microscopic Findings

Ultrathin sections of the agent-infected Vero cells were prepared and stained according to the method by Oyanagi et al. (1971). Nucleocapsid-like structures were found in the nuclei of syncytia. They were present also in the cytoplasm but much fewer in number. Figure 7 shows the nucleocapsids in a nucleus. The structure of filamentous form aggregated in boundles or scattered diffusely. In cross section, the former gave an appearance of crystalline array. Individual nucleocapsids, at a high magnification, had hollow structures. The outer diameter of the structure was 12 to 16 mμ and the inner diameter approximately 6 mμ. On the outer surface, striations with periodicity of 5.5 mμ were observed. The filamentous structures in the cytoplasm had a somewhat different appearance from the above, with fine granular materials

attached to their outer surface. In this regard the findings were essentially the same as those described by Oyanagi et al. (1971) on SSPE viruses. No particles suggesting virions of paramyxovirus could be recognized, nor were seen in nucleus or cytoplasm papova-like virions, that were described by Barbanti-Brodano et al. (1970) and discussed by Koprowski et al. (1970) as viruses hypothetically associated with SSPE agent.

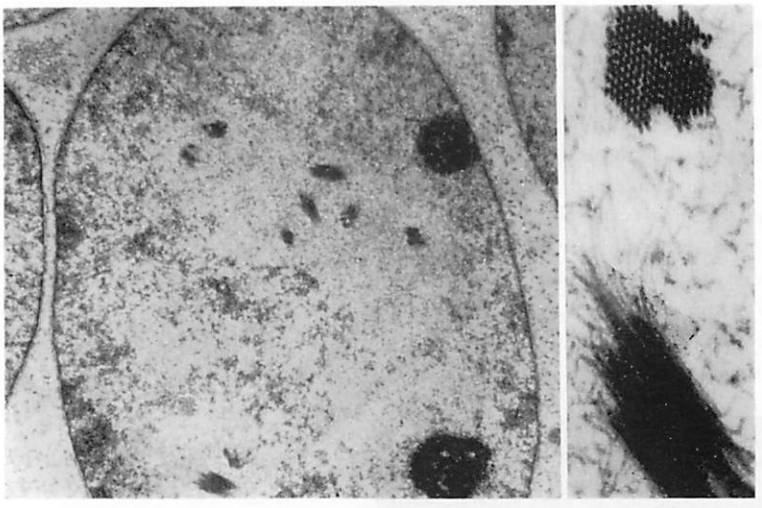

Fig. 7. Multinucleated giant cell of the 25th SSPE-Vero cell coculture. Left; Accumulations of nucleocapsids in the nucleus and cytoplasm are seen (×6,200). Right; Higher magnification of nucleocapsids in the nucleus (×31,000).

Effect of Actinomycin D and BUDR on Plaque Formation of SSPE Agent

As shown in Table II, an enhancing effect of actinomycin D on the formation of SSPE plaque is evident. BUDR did not decrease the plaque counts, in contrast to the results on herpes simplex virus used as the control. The overall results on the SSPE agent are consistent with those of measles virus.

Superinfection of SSPE Cultures with Measles and Type 1 Polio Viruses

Enlargement of the SSPE plaques stopped when they reached certain sizes. To elucidate the mechanism of this phenomenon, possible interference with superinfecting viruses was tested. Measles virus and type 1 poliovirus were chosen as the challenge viruses. Table III shows absence of interference with either virus. Although the virus yields fluctuated to some extent, it was interpretable by the small number of tube cultures per dilution used in the infectivity titrations. It should be taken into consideration that comparable yields of superinfecting viruses were obtained even from the SSPE monolayers of later stages which had many secondary small plaques and, hence, fewer normal cells in comparison with the control Vero cell monolayers. Possibility of rescue of the incomplete SSPE agent by the superinfecting measles virus was not investigated.

TABLE II

Effects of actinomycin-D and BUDR on plaque formation of SSPE agent

Agent tested	Plaque counts per bottle of groups			
	A Act-D	B BUDR	C BUDR+TDR	D Control
SSPE	142	24	23	26
MV	156	38	37	36
HSV	158	36	217	208

Abbreviations: MV=measles virus; HSV=herpes simplex virus; Act-D=actinomycin D; TDR=thymidine.

Monolayers of Vero cells in 2-oz bottles were inoculated with the SSPE cells, measles virus or herpes simplex virus. After an adsorption period of 1 hr at 37 C, the bottles of each group were divided into four groups (A, B, C and D). The SSPE bottles were overlaid with liquid medium; other virus-inoculated bottles with the agar overlay (Difco Noble agar, 1.0 %). The overlay media contained actinomycin D (0.1 μg/ml) for group A, BUDR ($10^{-4.3}$ M) for group B, BUDR ($10^{-4.3}$ M) plus TDR (10^{-3} M) for group C and no additives for group D. After incubation at 36 C for 6 to 7 days, plaque counts were made on the stained monolayers.

TABLE III

Propagation of measles virus and type 1 poliovirus in SSPE cell cultures

Challenge virus	Cell cultures	Yield of virus in cell cultures challenged on postseeding day of				
		1	3	5	7	9
Measles	SSPE-Vero	6.3	6.3	5.5	6.3	6.5
	Vero only	6.5	6.0	6.5	6.5	6.3
Polio-1	SSPE-Vero	6.7	7.3	7.0	7.3	7.0
	Vero only	6.5	7.2	7.3	7.3	7.7

The SSPE-Vero cell cocultures were started in 4-oz bottles using SSPE cell suspension containing about 160 plaque-forming cells (3rd postseeding day-reading). On the indicated days, each of 2 cultures of each group was challenged with measles virus ($10^{6.5}$ TCD_{50}) or type 1 poliovirus ($10^{4.3}$ TCD_{50}). After the adsorption and then removal of the inocula by washing, 12 ml of maintenance medium was added and the cultures were incubated at 36 C for 2 days (measles virus) or one day (poliovirus). The centrifugal supernatant fluid from the frozen-thawed cultures were titrated for infectivity on Vero cell cultures, 4 tubes per each of ten-fold dilutions. The control Vero cell cultures were prepared, challenged and treated in parallel with the SSPE cultures. Yield of virus was expressed in terms of log TCD_{50} per 0.2 ml.

Trials for Isolation of Cell-free Virus

Isolation of cell-free viruses was attempted by inoculating the test materials into Vero cell cultures. The culture fluid and the frozen-thawed or sonicated cell extracts were tested repeatedly during our maintenance up to 60th passage of the SSPE strain.

All trials gave negative results (Table IV). Care was taken to remove cells or cell debris by centrifugation before testing the samples.

TABLE IV

Trials for isolation of cell-free virus

Source of material for test	Special treatment given to the source culture system	Material tested
SSPE-Vero cell cocultures	None	Culture fluid
	None	Frozen-thawed cell extract
	None	Sonicated cell extract
	Incubated in the medium added with trypsin[1)]	Culture fluid
	Fused with UV-irradiated Sendai virus[2)]	Culture fluid & frozen-thawed cell extract
	UV-irradiation of SSPE cells before mixing with fresh Vero cells[3)]	Culture fluid
Infected guinea pig brain-Vero cell cocultures[4)]	None	Culture fluid & frozen-thawed cell extract

1) Trypsin 1:250 (Difco) was added to the cocultures on the next day of the seeding by replenishing with maintenance medium containing 1.5 μg/ml of trypsin and incubation was continued (Itoh et al., 1970). Formation of SSPE plaque was enhanced in its size and number. Culture fluids harvested on the 3rd day of incubation were tested.
2) The potent dilution of the fusion factor (UV-inactivated parainfluenza type 1, Sendai virus (Okawa et al., 1970) was applied to the coculture monolayer on the next day of the seeding. The fused cultues were incubated. The test materials were harvested at various times of incubation.
3) UV-irradiation for 30 or 60 seconds was made on SSPE infected cell suspensions under controlled conditions. The irradiated cells were mixed with fresh Vero cells and cocultivated. Thirty seconds' irradiation reduced plaque number by 27 % and 60 seconds' irradiation by 62 %. Culture fluids harvested at various times were tested.
4) SSPE cells recovered from the brain of a guinea pig infected by intracerebral inoculation were passaged four times by cocultivation with Vero cells. Culture fluids and cell extracts of the 4th passage were tested at various times of incubation.

Freezing Storage of SSPE Cells

For the purpose of maintenance of the agent, freezing storage at −70 C as used for Vero cell line was examined on the SSPE cells. The suspending medium was added with DMSO in 10 %. As shown in Table V, the storage was successful and gave essentially the same results as with uninfected Vero cells. This finding may suggest an intimate association of the present agent with the host cells.

Intracerebral Inoculation into Experimental Animals

Adult guinea pigs, mice and hamsters were inoculated intracerebrally with varying numbers of the SSPE cells. The representative results are shown in Tables VI to VIII. These animals showed neurologic signs following the inoculation and most of

TABLE V

Freezing storage of SSPE cells with DMSO

Days of storage	Plaque counts recovered	Percent recovery
Before freezing	268	—
1	148	55
14	139	52
28	126	47
56	116	43
98	108	40

A suspension of 3.5×10^6 cells/ml in the growth medium containing 10% DMSO was made from the SSPE-Vero cell cocultures. The suspension was distributed into ampoules in 1.0-ml amounts. The ampoules were sealed and stored frozen at −70 C in the electrically regulated freezer. On the indicated days, 2 ampoules were quickly thawed. The contents were suspended with fresh Vero cells to make 60 ml in the medium, distributed into five 4-oz bottles and cultivated at 36 C. From the counting made on the 3rd day after seeding plaque numbers per ampoule were calculated.

TABLE VI

Intracerebral inoculation of guinea pigs with SSPE cells

Inoculum		Pre-inoculation measles virus antibody[2]	Mortality	Incubation period in days
Cells	Plaques[1]			
SSPE	1,800	<8	5/5	6—10
SSPE	1,800	>2,048	0/5	—
Measles virus-infected Vero cells	530,000[3]	<8	0/5	—

1) Plaques were counted after 3 days of cocultivation of the inoculum with fresh Vero cells.
2) Pre-inoculation sera were tested for hemagglutination-inhibition antibodies with the standardized measles virus antigen and cercopithecus monkey erythrocytes.
3) Infected cells in the inoculum were counted by enumeration of cells showing specific immunofluorescence in the smear stained with the FITC-labeled anti-measles rabbit serum globulin.

them died in various periods. However, the susceptibility to the infection was different from one animal species to another. Under our conditions, mice and hamsters seemed to be more susceptible than guinea pigs in which the incubation periods tended to be longer even with larger numbers of infected cells for inoculation. Table VI shows the results on guinea pigs. It was demonstrated that preimmunization with measles virus protected the animals against the disease and that the measles virus-infected cells, even in far larger numbers, did not cause neurologic signs. In Fig. 8, a neuron-like cell having specific fluorescent granules in its processes is shown. Similar figures were frequent in the brains of infected guinea pigs. Re-isolation of SSPE-infected cells from the guinea pig brains was repeatedly successful. As an example, more

TABLE VII

Intracerebral inoculation of adult mice with SSPE cells

Inoculum size in terms of plaque number[1)]	Mortality	Incubation period in days
86	4/5	4 - 9
22	2/5	4 - 8
5.4	2/5	6 - 11
1.3	2/5	8 - 11
0.3	0/5	—

1) See footnotes to Table VI.

TABLE VIII

Intracerebral inoculation of adult hamsters with SSPE cells

Inoculum size in terms of plaque number[1)]	Mortality	Incubation period in days
30	6/6	5 - 6
12	2/6	5 - 6

1) See footnotes to Table VI.

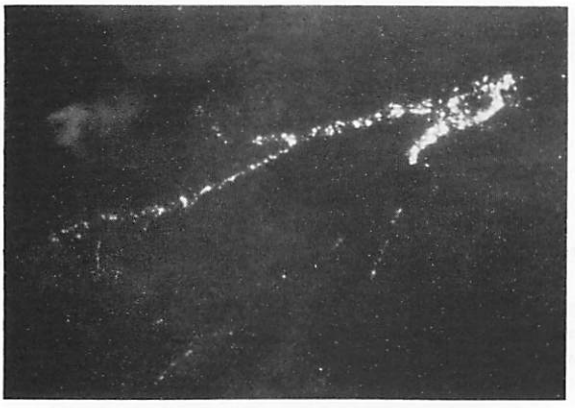

Fig. 8. A nerve cell of the brain of an infected guinea pig showing specific fluorescent granules in its dendrit processes.

than 20,000 plaque-forming cells were recovered from the brain of a guinea pig sacrificed on day 9 after inoculation of about 1,000 plaque-forming cells. In view of the demonstration of immunofluorescent granules in nerve cells as shown in Fig. 8, there remained little doubt that these recovered plaque-forming cells included infected host brain cells.

Discussion

In accord with the SSPE agents reported by others, the present agent has also been shown to possess antigenicity of measles virus. However, its characteristic feature

is such a close association with host cells that no cell-free virus has been demonstrated under various conditions as described in the text. Absence of matured virus particles under the electron microscopic observations and the long-term preservation of the agent-harvoring cells in the frozen state coincided with the characteristic property. In regard to intercellular spreading of the agent, its direct cell-to-cell transmission by a fusion process resulting in the syncytium formation is most likely. Reduction in number and size of the plaques by measles virus antiserum may be acceptable, if we assume that mechanism of the syncytium formation could be affected by the specific antibodies which react on the modified cell membrane of SSPE cell. It is improbable that cell division is a principal way for the cell-to-cell spreading because mitotic figures were absent in the syncytia and the plaques appeared so early, ten hours after the seeding, that multiple cycles of mitosis are unlikely.

Destruction of the monolayer by the present agent was not so progressive as that by measles virus. Enlargement of plaquas stopped at certain limited sizes and they were very circular when the cultures were incubated undisturbed. It seemed to us that a certain type of interference worked in the cocultures, especially for the intact cells just adjacent to the syncytia. An attempt at demonstrating production of a soluble inhibiting factor by the SSPE cultures was carried out with the whole culture extract as the test material and Vero cell cultures as the host substrate. The 24-hour treated Vero cell monolayer did not inhibit the plaque formation as compared with the control untreated monolayer, thus denying accumulation of soluble inhibitors in the SSPE cultures. Tests for interference with polio or measles virus in the SSPE cultures were made to see whether other types of interference participate in this phenomenon. As shown in the text, no suppression of the yields of superinfecting viruses was found. Incidentally, the latter results with measles virus made it clear that precautions against a laboratory contamination with measles virus are of primary importance for the maintenance of the SSPE agent.

In animal experiments, intracerebral inoculations of the infected cells induced similar neurologic diseases in adult guinea pigs, mice and hamsters. The susceptibility was different from one animal species to another. Guinea pigs were less susceptible than mice and hamsters. It is our impression that animals develop experimental diseases so easily is another characteristic feature of the present strain.

Experimental models in animals of human SSPE are subjects to be sought for. It is difficult to consider that the neurologic diseases brought about by an artificial procedure, such as intracerebral inoculation of agent-harvoring cells, have pathogenesis common with natural diseases in humans. But it is apparent from our histological examinations that host brain cells were involved in the experimental infections (Sato et al., 1972). Besides, specific immunofluorescent granules were found in nerve cells as shown in Fig. 8. Mechanisms of transmission of the agent to neurons are of interest and their elucidation will contribute to the study of human SSPE.

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Size, Composition, and Structure of the Deoxyribonucleic Acid of Herpes Simplex Virus Subtypes 1 and 2

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Studies of the size, composition, and structure of the deoxyribonucleic acid (DNA) of the F and G prototypes of herpes simplex virus (HSV) subtypes 1 and 2 (HSV-1 and HSV-2) showed the following. (i) As previously reported by Goodheart et al. HSV-1 and HSV-2 DNA have a buoyant density of 1.726 and 1.728 g/cm^3, corresponding to 67 and 69 guanine ± cytosine moles per cent, respectively. The difference in guanine plus cytosine content of the DNA species was confirmed by the finding of a 1 C difference in T_m. (ii) The DNA from purified virus on cocentrifugation with T4 DNA in neutral sucrose density gradients sedimented at 55*S*, corresponding to 99 ± 5 million daltons in molecular weight. HSV-1 and HSV-2 DNA could not be differentiated with respect to size. (iii) Cosedimentation of alkali-denatured DNA from purified virus with T4 DNA on alkaline sucrose density gradients consistently yielded several bands of single-stranded HSV DNA ranging from fragments 7 × 10^6 daltons to intact strands 48 × 10^6 daltons in molecular weight.

Naturally occurring herpes simplex viruses (HSV) differ in a number of biological, physical, and immunological properties (5, 7, 8, 10, 17-19, 20, 27). As determined in neutralization tests, most [but not all (31)] fresh isolates fall into two subtype groups designated as 1 (HSV-1) and 2 (HSV-2).

This report concerns the properties of HSV deoxyribonucleic acid (DNA). Previous reports have estimated the molecular weight of HSV-1 DNA to be 68 × 10^6 (24) to 100 × 10^6 daltons (2) and to contain 65 to 68 per cent guanine plus cytosine (10, 11, 20, 22, 23). HSV-2 DNA was reported (10) to contain 70.4 moles per cent guanine plus cytosine, and nothing was known of its size or relatedness to HSV-1 DNA. In the experiments described in this paper, we have directly compared the size, composition, and structure of HSV-1 and HSV-2 DNA.

MATERIALS AND METHODS

Solutions. Virus buffer consisted of 0.15 M NaCl and 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.5. Neutral DNA buffer consisted of 1 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA), and 0.05 M Tris-hydrochloride, *p*H 7.5. Alkaline DNA buffer consisted of 0.8 M NaCl, 0.3 M NaOH, and 0.001 M EDTA. Reticulocyte standard buffer (RSB) consisted of 0.01 M sodium chloride, 0.001 M magnesium chloride, 0.01 M Tris, *p*H 7.5. Standard saline citrate (1 × SSC) consisted of 0.15 M sodium chloride, 0.015 M sodium citrate. SDS buffer consisted of 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris, *p*H 7.4.

Cells. HEp-2 and Vero cells were originally obtained from Flow Laboratories, Rockville, Md., and were grown in minimal essential medium supplemented with 10% calf serum.

Virus and infection of cells. The properties of the F and G prototypes of HSV-1 and HSV-2 have been previously described (7). The procedures used for infection of HEp-2 and Vero cells with HSV-1 and HSV-2 were those described previously for infection of HEp-2 cells with HSV-1 (7).

Labeling of cells with radioactive isotopes. HEp-2 cells infected with 20 plaque-forming units (PFU) of HSV-1 or HSV-2 per cell were labeled between 4 and 20 hr postinfection with 10 μCi of ^{3}H-thymidine or 0.1 μCi of ^{14}C-thymidine (Schwarz-Mann, Orangeburg, N.Y.) per ml of medium of mixture 199 lacking thymidine and supplemented with 1% dialyzed calf serum.

Purification of virus: preparation of nucleocapsids. Cells infected with HSV-1 or HSV-2 were harvested by scraping and centrifuged at 800 × *g* in a PR-2 refrigerated centrifuge for 10 min at 4 C. The cell pellet was washed with phosphate-buffered saline (PBS) and resuspended in RSB containing 0.5% Nonidet P-40 (Shell Chemical Co., New York, N.Y.) for 10 min at 4 C. The cells were then homogenized with six

strokes of a tight fitting Dounce homogenizer, and the nuclei were removed by centrifugation of the homogenate at 800 × *g* for 10 min at 4 C in the PR-2 centrifuge. The cytoplasm was layered onto 37-ml linear 10 to 50% (w/w) sucrose density gradients, prepared in virus buffer and centrifuged for 1 hr at 25,000 rev/min in the SW27 rotor at 5 C.

Purification of virus: preparation of enveloped virus. The preparation of enveloped HSV-1 followed a procedure of Spear and Roizman (*manuscript in preparation*). Briefly, cells infected with HSV-1 were harvested and washed as described above. The cell pellet was resuspended in 0.01 M Tris-hydrochloride (*p*H 7.4) for 10 min at 4 C, homogenized with four strokes of a tight fitting Dounce homogenizer, and adjusted to 0.25 M sucrose by the addition of one-seventh volume of 2 M sucrose in 0.01 M Tris-hydrochloride (*p*H 7.4). The nuclei were removed by centrifugation for 10 min at 800 × *g* in a PR-2 refrigerated centrifuge. The cytoplasm was layered onto 17-ml linear dextran 10 gradients (1.04 to 1.09 g/cm^3) and centrifuged for 1 hr at 25,000 rev/min and 5 C in a SW 25.3 rotor.

Determination of relative sedimentation rates of HSV-1 and HSV-2 DNA: preparation of ^{14}C-labeled T4 DNA. T4 phage prepared in *E. coli* and labeled with ^{14}C-uridine was the kind gift of Robert Haselkorn. The phage was resuspended in 0.5% sodium dodecyl sulfate (Matheson Scientific Co., Elk Grove Village, Ill.) and 2% sarkosyl NL97 (Geigy Chemical Co., Ardsley, N.Y.) in neutral DNA buffer and gently rolled with phenol at 60 C for 2 min. The phenol phase was removed, and the DNA was gently rolled with chloroform-isoamyl alcohol (2%, v/v) until the aqueous phase was clear.

^{3}H-thymidine-labeled simian virus 40 form I DNA (obtained from S. Kit and D. M. Dubbs) and ^{3}H-thymidine-labeled *E. coli* plasmid DNA (obtained from N. Cozarelli) were used to determine the relative sedimentation constant of the purified T4 DNA in alkaline sucrose gradients.

Sedimentation in neutral sucrose gradients. ^{3}H-thymidine-labeled HSV-1 or HSV-2 nucleocapsids were disrupted by exposure to 0.5% sodium dodecyl sulfate, 2% sarkosyl in neutral DNA buffer at 60 C for 2 min; mixed with ^{14}C-labeled T4 DNA; poured onto 12-ml linear 10 to 30% (w/w) sucrose gradients prepared in neutral DNA buffer; and sedimented for 3.5 hr at 40,000 rev/min in an SW41 rotor at 20 C. Fractions (0.3 ml) were collected from the top of the gradients, and the DNA was precipitated onto filters (HAWP 25-mm; Millipore Filter Corp., Bedford, Mass.) with 5% trichloroacetic acid by using herring sperm DNA (Sigma Chemical Co., St. Louis, Mo.) as a carrier. The filters were dried and assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer.

Sedimentation in alkaline sucrose gradients. ^{3}H-thymidine-labeled HSV-1 or HSV-2 nucleocapsids were mixed with ^{14}C-labeled T4 phage and resuspended in 0.5% sodium dodecyl sulfate and 2% sarkosyl in alkaline DNA buffer. The DNA species were poured onto 12-ml linear 10 to 30% sucrose gradients prepared in alkaline DNA buffer and sedimented for 3.5 hr at 40,000 rev/min in an SW41 rotor at 20 C.

The procedures for the collection of sucrose density gradient fractions, precipitation of the DNA, and assay of radioactivity were as described above.

Extraction and sedimentation of viral DNA from whole cytoplasmic lysate and from purified enveloped virus. HEp-2 cells were infected with 40 PFU of HSV-1 per cell and labeled from 4 hr postinfection with 10 μCi of ^{3}H-thymidine per ml of medium. At 16 hr postinfection, the cells were harvested, allowed to swell in 0.01 M Tris (pH 7.4) and 2.5 mM EDTA, and then Dounce-homogenized as previously described. A sample of the cytoplasm was layered on a dextran 10 gradient and centrifuged to obtain enveloped virus. The DNA was extracted from enveloped virions and cosedimented with T4 DNA as described above. Another sample of the cytoplasm was dissolved in 0.5% sodium dodecyl sulfate, 2% sarkosyl in neutral DNA buffer at 60 C for 2 min; mixed with ^{14}C-labeled T4 DNA; and cosedimented in neutral sucrose density gradients. Finally, a sample of cytoplasm and a sample of purified enveloped virus harvested from the dextran 10 gradient were each mixed with T4 phage labeled with ^{14}C in the DNA; solubilized in 0.5% sodium dodecyl sulfate, 2% sarkosyl in alkaline DNA buffer; and centrifuged in alkaline sucrose density gradients. All centrifugations were done as described above.

Purification of viral DNA for isopycnic banding and thermal denaturation. Purified herpes simplex nucleocapsids were resuspended at 60 C in neutral DNA buffer containing 0.5% sodium dodecyl sulfate and 2% sarkosyl, gently poured onto 12-ml linear 10 to 30% sucrose gradients, and centrifuged in an SW41 rotor for 3.5 hr at 40,000 rev/min and 20 C. Fractions (0.3 ml) were collected through a flow cell continuously monitored for absorbance at 260 nm. The fractions containing intact HSV DNA were dialyzed against 0.1× SSC, digested with 50 μg of heat inactivated pancreatic ribonuclease (Schwarz-Mann, Orangeburg, N.Y.) per ml, adjusted to 1.5× SSC, and further digested with 50 μg of ribonuclease per ml. Sodium dodecyl sulfate was added to make a final concentration of 0.5%, and the protein was extracted by rolling the DNA with phenol and chloroform-isoamyl alcohol. The DNA was then dialyzed four times for 24 hr against 100 volumes of 0.1× SSC to remove traces of phenol.

Isopycnic banding of HSV DNA in cesium chloride. Purified HSV-1 or HSV-2 DNA was mixed with 2 μg of SP01 DNA (gift of K. Bott), dialyzed against cesium chloride at a density of 1.715 g/cm^3 in 0.01 M Tris-hydrochloride (*p*H 7.4), and centrifuged in the Spinco model E analytical centrifuge at 44,700 rev/min and 25 C for 20 hr. Ultraviolet (UV) absorption photographs were scanned with the Joyce Loebel microdensitometer.

Melting temperature of HSV DNA. Purified HSV-1 DNA and HSV-2 DNA in 0.1× SSC were dialyzed against 0.1× SSC in the same flask to eliminate possible artifacts atributable to differences in salt concentration. The thermal denaturation profiles of the two DNA species were determined simultaneously in 0.5-ml Teflon-stoppered quartz cuvettes with a 1-cm light path (Hellma Cells Inc., Jamaica, N.Y.) by using a Gilford recording spectrophotometer equipped with

a linear temperature programmer and internal thermistor (16).

RESULTS

Viral preparations: naked nucleocapsids. Nonidet P-40-extracted cytoplasm of 20 hr-infected HEp-2 or Vero cells yielded (2) prominent bands on centrifugation through a 10 to 50% (w/w) sucrose density gradient. Electron microscopy of negatively stained preparations showed that the top band contained a mixed population of partially disrupted, intact, empty, and full nucleocapsids. The bottom band contained predominantly full nucleocapsids contaminated with small amounts of membrane debris.

The bottom band from the sucrose gradients was used as the source of DNA from nucleocapsid preparations.

Enveloped virus preparations. The cytoplasm of HSV-1-infected cells on centrifugation through a dextran 10 density gradient as described above yielded a single band in the middle of the tube. Electron microscopic analysis indicated that the band contained fully enveloped virus impermeable to phosphotungstic acid and was free from partially disaggregated virions. This finding confirms numerous other observations that the cytoplasm of cells infected with HSV-1 virus contains predominantly enveloped nucleocapsids (28).

Isopycnic banding of HSV-1 and HSV-2 DNA in neutral CsCl. The density of HSV-1 and HSV-2 DNA in neutral CsCl was determined in the Spinco model E centrifuge with SP01 as an internal marker as described above. The results are shown in Fig. 1. The densities of HSV-1 DNA and HSV-2 DNA calculated according to Szybalski (30) with the value of 1.742 g/cm³ for the density of SP01 DNA are 1.726 ± 0.0005 and 1.728 ± 0.0005 g/cm³, respectively.

Thermal denaturation of HSV-1 and HSV-2 DNA. The UV absorption-thermal denaturation profile of HSV-1 and HSV-2 DNA was determined in 0.1× SSC as described above. The results shown in Fig. 2 indicate that the melting temperatures of HSV-1 and HSV-2 DNA in 0.1× SSC are 82 and 83 C, respectively.

Determination of relative sedimentation rates and molecular weight of HSV-1 and HSV-2 DNA: sedimentation of native DNA. The sedimentation rate of HSV-1 and HSV-2 DNA in neutral sucrose density gradients was compared with that of T4 DNA as described above. The results shown in Fig. 3 indicate the following.

(i) Both HSV-1 and HSV-2 DNA extracted from nucleocapsids with sodium dodecyl sulfate and sarkosyl and centrifuged in neutral sucrose density gradients form a single sharp band slightly above that of T4 DNA (Fig. 3A, B). Identical

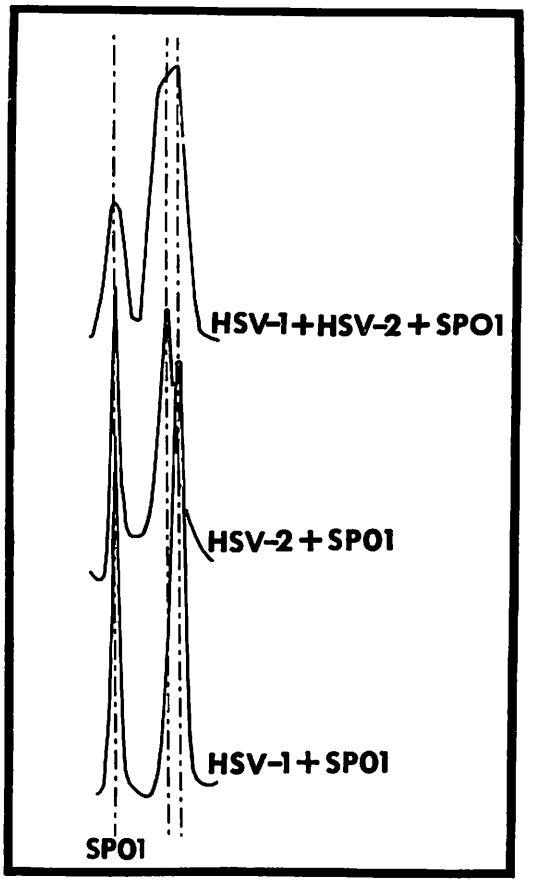

FIG. 1. *Buoyant density determination of HSV-1 DNA, HSV-2 DNA, and of an artificial mixture of HSV-1 and HSV-2 DNA determined by centrifugation with SP01 DNA in a model E analytical centrifuge as described in Materials and Methods. The UV absorption photograph was scanned with a Joyce Loebel microdensitometer.*

results were obtained when HSV nucleocapsids were mixed with T4 phage and coextracted by gently rolling with phenol and chloroform-isoamyl alcohol.

(ii) T4 DNA used in this study was intact double-stranded DNA by the following criteria. T4 DNA formed a single discrete band in neutral sucrose gradients. Alkali denaturation of the T4 DNA yielded a single discrete band in alkaline sucrose gradients. Cosedimentation of T4 DNA with simian virsus 40 form I DNA and *Escherchia coli* plasmid DNA in alkaline sucrose gradients demonstrated that the sedimentation rate of the denaturated T4 DNA was identical to that previously reported (3, 14).

(iii) The ratio of the distances sedimented by HSV-1 and HSV-2 DNA to that sedimented by

T4 DNA was 0.96 ± 0.02. The sedimentation coefficient of HSV-1 DNA and HSV-2 DNA in neutral sucrose density gradients derived from the relationship of Burgi and Hershey (4), $(d_1/d_2) = (s_1/s_2)$, was 55S. The molecular weight of HSV DNA was determined from the equation $s_1/s_2 = (M_1/M_2)^{0.38}$ (4, 9). With the value of 110 × 10^6 daltons for the molecular weight of T4 DNA (1, 6, 26), the molecular weight of HSV DNA was calculated to be 99 ± 5 × 10^6 daltons.

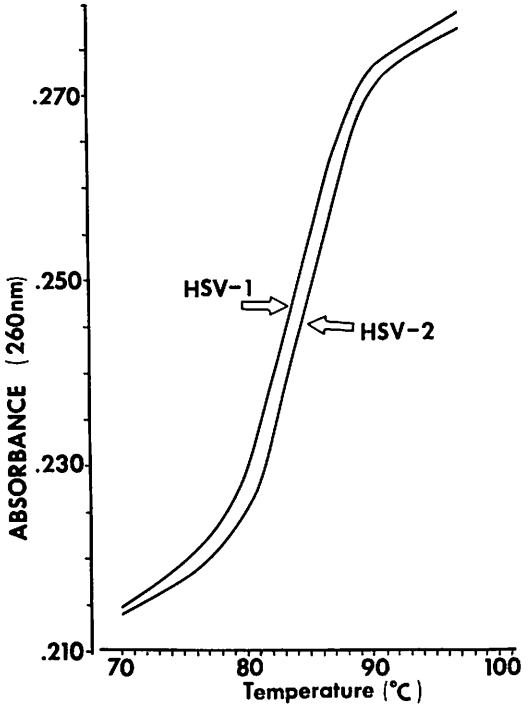

FIG. 2. *UV absorbance-thermal denaturation profile of HSV-1 and HSV-2 DNA in 0.1× SSC, determined as described in Materials and Methods.*

(iv) Cosedimentation of HSV-1 and HSV-2 DNA in neutral sucrose gradients (Fig. 3C) indicated that the sedimentation coefficients of these DNA species cannot be differentiated by zone centrifugation.

Sedimentation of denatured DNA. The sedimentation rate of denatured HSV-1 and HSV-2 DNA was compared to that of denatured T4 DNA in alkaline sucrose density gradients as described above. Figure 4 shows the distribution of DNA in the gradients. The results indicate the following.

(i) HSV-1 and HSV-2 DNA formed multiple bands in alkaline sucrose density gradients. The formation of multiple bands is not an artifact of the procedures used in the purification of the virus or in the extraction and the sedimentation of DNA. The DNA extracted from enveloped HSV-1 showed a similar distribution in alkaline sucrose gradients. The distribution of DNA bands was unaffected by the presence of 2.5 mM EDTA during all stages of viral purification. T4 DNA coextracted with HSV-DNA from mixtures of HSV nucleocapsids and T4 phage yielded a single band on sedimentation in alkaline sucrose. Mixtures of T4 phage and HSV nucleocapsids lysed directly on the top of alkaline gradients yielded similar results to those shown in Fig. 4A and B.

(ii) The most prominent and most rapidly sedimenting band of HSV-1 and HSV-2 DNA localized slightly above T4 DNA (Fig. 4A, B). The ratio of the distances of the sedimentation of HSV DNA to that of T4 DNA was 0.93. On the basis of this ratio, the most promient HSV band was estimated to be 68S. With the equation and coefficients of Studier (29), we estimate the molecular weight of HSV DNA in the rapidly sedimenting band to be 49 × 10^6 daltons. Co-centrifugation of HSV-1 and HSV-2 indicated that the sedimentation coefficients of the most prominent

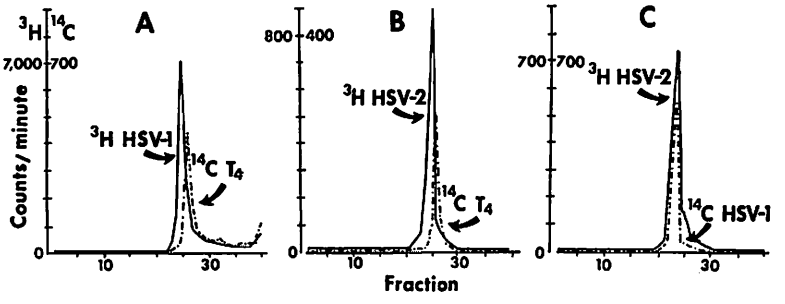

FIG. 3. *Zone sedimentation of HSV DNA in neutral sucrose density gradients. (A) Co-centrifugation of ^{3}H-HSV-1 DNA with ^{14}C-T4 DNA. (B) Co-centrifugation of ^{3}H-HSV-2 DNA with ^{14}C-T4 DNA. (C) Co-centrifugation of ^{3}H-HSV-2 DNA with ^{14}C-HSV-1 DNA. The DNA species were centrifuged for 3.5 hr in an SW41 rotor at 40,000 rev/min and 20 C. Symbols: dashed line, ^{14}C-labeled DNA; solid line, ^{3}H-labeled DNA. Direction of sedimentation is to the right.*

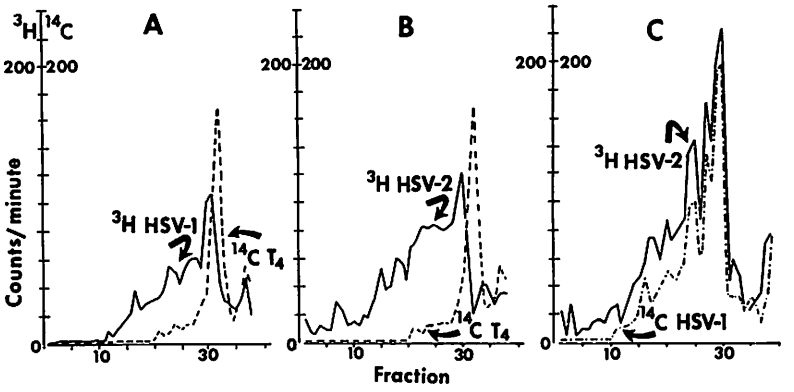

FIG. 4. *Zone sedimentation of alkali denatured HSV DNA and T4 DNA in alkaline sucrose density gradients. (A) Co-centrifugation of ^3H-HSV-1 DNA with ^{14}C-T4 DNA. (B) Co-centrifugation of ^3H-HSV-2 DNA with ^{14}C-T4 DNA. (C) Co-centrifugation of ^3H-HSV-2 DNA with ^{14}C-HSV-1 DNA. The DNA species were centrifuged for 3.5 hr in an SW41 rotor at 40,000 rev/min and 20 C. Symbols: dashed line, ^{14}C-labeled DNA; solid line, ^3H-labeled DNA. Direction of sedimentation is to the right.*

band of the two DNA species cannot be differentiated by zone centrifugation in alkaline sucrose density gradients.

(iii) With respect to the slower sedimenting species of HSV-1 and HSV-2 DNA the data are as follows. At least three discrete bands are usually found, ranging in ratio of distances sedimented relative to T4 DNA of 0.51 to 0.84, corresponding to a range in molecular weights of 7×10^6 to 30×10^6 daltons. Some variability in the distribution of the DNA in alkaline sucrose gradients was found between different preparations of the same virus. The more slowly sedimenting species constitute at least half the total viral DNA.

(iv) Cosedimentation of HSV-1 or HSV-2 DNA extracted from nucleocapsids grown in Vero cells with T4 DNA yielded a similar distribution to that seen in Fig. 4A and B.

Structural identity of viral DNA in crude cytoplasmic lysate and in purified enveloped virus. These experiments were designed to determine whether the single-stranded breaks in viral DNA were a constant feature of purified virions or whether similar breaks were present in crude cellular extracts containing virions. The size of the labeled native and denatured DNA in the whole cytoplasm and in the enveloped virus band obtained from HEp-2 cells infected for 16 hr with HSV-1 was determined in neutral and alkaline sucrose density gradients as described above. The results shown in Fig. 5 indicate the following.

(i) Labeled DNA released by sodium dodecyl sulfate and sarkosyl from virions in the cytoplasm of cells infected for 16 hr with HSV-1 (Fig. 5A) sedimented in neutral sucrose density gradients exactly like the DNA extracted from purified virions. The ratio of the distances sedimented by the cytoplasmic DNA relative to that of T4 DNA was 0.96%, i.e., the same as found in the results of the experiment summarized in Fig. 3.

(ii) Cosedimentation of denatured DNA extracted from the cytoplasm (Fig. 5B) or from enveloped virus (Fig. 5C) with T4 DNA in alkaline sucrose density gradients yielded identical results, indicating that enveloped virus accumulating in the cytoplasm contains DNA with single-stranded nicks and that the breaks are not the consequence of manipulations involved in virus purification.

DISCUSSION

These studies were undertaken to compare the size and structure of the DNA of the two major subtypes of herpes simplex virus. The data obtained in these studies are summarized in Table 1.

Composition of HSV-1 and HSV-2 DNA. Previous estimates of the buoyant density of HSV-1 and HSV-2 DNA were based on comparative distances between cellular and viral DNA bands of HSV-1 and HSV-2 infected cells (10). These studies indicated that HSV-2 DNA had a higher buoyant density than HSV-1 DNA. With SP01 DNA as an internal marker, we obtained values of 1.726 and 1.728 g/cm^3 for the buoyant density of HSV-1 and HSV-2, respectively (Table 1). Based on the assumption that there are no unusual bases in HSV-1 and HSV-2 DNA, the mole percentages of guanine plus cytosine bases are estimated to be 67 and 69% (25). UV absorbancy-thermal denaturation studies indicated a 1 C difference in the T_m of HSV-1 and HSV-2 DNA as expected for DNA species differing by 2 moles per cent guanine plus cytosine.

Size of HSV-1 and HSV-2 DNA. Previous estimates of the molecular weight of HSV-1 DNA

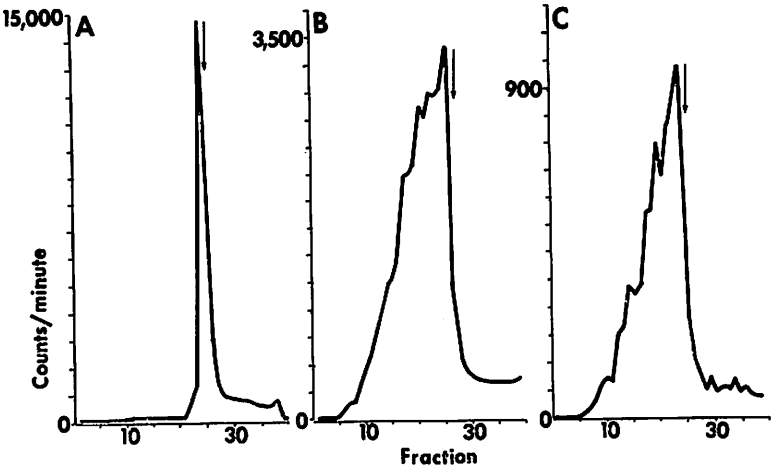

FIG. 5. *Zone sedimentation in neutral and alkaline sucrose density gradients of HSV DNA extracted from crude cytoplasm of infected cells and from purified enveloped virus in that cytoplasm. (A) Native ^{3}H-HSV-1 DNA in the crude cytoplasm sedimented in neutral sucrose gradients. (B) Denatured ^{3}H-HSV-1 DNA in alkaline sucrose gradients. (C) Denatured ^{3}H-HSV-1 DNA extracted from enveloped virions purified from the crude cytoplasmic lysate by sedimentation through dextran 10 density gradient. Centrifugation was for 3 hr in an SW41 rotor and at 40,000 rev/min and 20 C. The position of ^{14}C-T4 DNA cosedimented with ^{3}H-HSV-1 DNA is indicated by arrow. Direction of sedimentation is to the right.*

TABLE 1. *Comparison of size and structure of the two major subtypes of herpes simplex virus (HSV-1 and HSV-2)*

Virus	Native DNA				Single-strand DNA		
					Intact strand		Overall range of fragments[c]
	S^a	Mol wt (daltons)	Buoyant density (g/cm^3)	Guanine cytosine moles per cent	S^b	Mol wt (daltons)	Mol wt (daltons)
HSV-1 (F)	55	99 ± 5[d]	1.726 ± 0.0005	67	68	48[d]	7–48[d]
HSV-2 (G)	55	99 ± 5	1.728 ± 0.0005	69	68	48	7–48

[a] In neutral sucrose relative to T4 DNA.
[b] In alkaline sucrose relative to T4 DNA.
[c] Viral DNA from 16-hr-old infected cells.
[d] Expressed $\times 10^6$.

were obtained by two techniques. One involving direct measurement of the sedimentation coefficient of the DNA yielded a molecular weight of 68 $\times$ 10^6 daltons (24) and is in all probability a low value. The second, using the Kleinschmidt technique, yielded an average value of 101 $\times$ 10^6 daltons with a range of 86 $\times$ 10^6 to 115 $\times$ 10^6 daltons (2). The size of HSV-2 DNA was unknown.

In principle, there are several techniques for determination of the absolute molecular weight of DNA molecules greater than 50 $\times$ 10^6 daltons: ^{32}P star enumeration (15), equilibrium sedimentation (26), and indirect determinations based on phosphate analysis and the molecular weight value of purified virions obtained by equilibrium sedimentation and sedimentation diffusion (1, 6). As reviewed by Friefelder (9), all of these techniques suffer from inherent errors. It is unlikely that DNA molecular weight determinations based on phosphate analysis and molecular weights of purified virions will ever be practical for the large enveloped DNA viruses. Other techniques, such as Kleinschmidt and sedimentation, depend on standardization against DNA species of known molecular weight. In regard to the Kleinschmidt technique, HSV presents an unusual problem. We are particularly concerned by recent observations (9, 12) that the mass per unit length for DNA of 65 to 70 guanine plus cytosine moles

per cent might be as much as 15% higher than that used in previous studies.

We chose to determine the molecular weight of HSV-1 and HSV-2 DNA by zone sedimentation in neutral sucrose density gradients by using T4 DNA as an internal standard for the following reasons. (i) Independent absolute determinations have been made for few DNA molecules, T4 being among them (1, 6, 26). (ii) T4 DNA is very close in size to HSV DNA. (iii) Recent data confirm that the Burgi and Hershey relationship is valid over small ranges of S and M (9).

The molecular weight of both HSV-1 and HSV-2 DNA was found to be $99 \pm 5 \times 10^6$ daltons.

Structure of HSV-1 and HSV-2 DNA. The molecular weight of denatured HSV-1 and HSV-2 DNA was obtained by zone centrifugation in alkaline sucrose gradients to determine whether the native DNA consisted of two continuous non-cross-linked single strands. The results indicated that, at most, only half of the single strands in any preparation of HSV-1 or HSV-2 DNA are intact; the remainder of the DNA is fragmented. Viral DNA in the cytoplasm of infected cells before isolation of purified virus had the same size distribution on alkali denaturation as the DNA of purified virus, effectively excluding the possibility that single-strand breaks arise during viral purification. The fragments of DNA, which range in size from 7×10^6 to 30×10^6 daltons, form several discrete bands in sucrose gradients, indicating that they are not products of random cleavage. No DNA was consistently found to be larger than intact single strands. Therefore, we conclude that native viral DNA contains single-strand breaks and that it is not cross-linked. Similar findings have recently been reported for Marek's disease virus DNA (13).

Significance of the single-strand fragments found in HSV DNA. Although the origin and biological significance of the single-strand fragments found in HSV DNA are not clear, several points should be made. (i) As noted previously, the fragments are not an artifact of the procedures used in viral purification or in extraction and analysis of the DNA. The alkaline denaturation experiments probably indicate the existence of breaks in the DNA, since alkali-labile linkages such as apurinic sites have not been found in viral DNA species. (ii) Both HSV-1 and HSV-2 DNA have been shown to have similar distributions of single-strand fragments. Furthermore, the finding is not a unique feature of virus growth in a particular cell line. (iii) The finding that DNA from purified virus yields on velocity centrifugation in alkaline sucrose gradients at least four bands ranging in size from 7×10^6 to 48×10^6 daltons suggests that the single-strand breaks are not random but are at specific sites. (iv) We do not know whether the fragments result from specific nicking of intact single strands or results from incomplete ligation of breaks necessary for control of transcription, as in T4 (21). These points are currently under investigation.

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Defectiveness of Interferon Production and of Rubella Virus Interference in a Line of African Green Monkey Kidney Cells (Vero)

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Vero cells, a line of African green monkey kidney cells, failed to produce interferon when infected with Newcastle disease, Sendai, Sindbis, and rubella viruses, although the cells were sensitive to interferon. Further, infection of Vero cells with rubella virus did not result in interference with the replication of echovirus 11, Newcastle disease virus, or vesicular stomatitis virus, even in cultures where virtually every cell was infected with rubella virus. Under the same conditions, BSC-1 cells and other cells of primate origin produced interferon and showed rubella virus interference. The results indicate that the presence of rubella virus in the cell does not in itself exclude multiplication of other viruses and that rubella virus interference appears to be linked to the capability of the cell to produce interferon.

Cytopathic changes are slight or absent in many kinds of mammalian cells infected with rubella virus, but the virus conveys resistance to superinfection with a large number of unrelated viruses (16). The interference does not affect the uptake and eclipse of the challenge viruses and thus resides at an intracellular level (11, 21). The interferon system represents the only known mechanism of heterologous viral interference which is characterized by these properties, and interferon has been found in tissue cultures infected with rubella virus (15, 17). These and other analogies have led some authors to suggest that rubella virus interference is mediated by the interferon system (5, 15, 26). However, other reports have indicated that the rubella virus genome may be directly responsible for the interference with Newcastle disease virus (10, 11). There also is the possibility that the activity of an interferon system in cells infected with rubella virus is merely coincidental or ancillary. The mechanism of rubella virus interference, whether or not it is mediated by the host cell genome, could be unrelated to interferon. The use of cells which are defective in the production of interferon or are insensitive to interferon could provide a clue as to whether rubella virus interference is basically dependent upon the interferon system. This study reports the finding that a stable line of African green monkey kidney cells (Vero) is defective in its production of interferon but is sensitive to the action of interferon, and describes the replication of certain viruses in such cells coinfected with rubella virus.

MATERIALS AND METHODS

Tissue culture. Vero cells, a stable line of African green monkey kidney cells (27), were kindly supplied by J. S. Rhim of Microbiological Associates, Bethesda, Md. (22) in their 140th passage and were used after 5 to 15 additional passages. BSC-1 cells (8), another stable line of African green monkey kidney cells, were obtained from laboratory stocks (19). Newborn foreskin fibroblasts were grown as described (4) and were used between passages 10 and 20. Primary chick embryo cell cultures were prepared from 10-day-old embryonated eggs. Primary African green monkey kidney cells (pGMK) were grown as described (13). With the exception of the latter cells (13), the growth medium consisted of Eagle's medium supplemented with 0.75 g of sodium bicarbonate per liter, 10% fetal bovine serum, 100 units of penicillin per ml, 100 μg of kanamycin per ml, and 50 units of mycostatin per ml. In maintenance medium, 1.5 g of sodium bicarbonate per ml and 2% fetal bovine serum were substituted. Agar overlays, 4 ml per 6-cm plastic petri dish, contained the same constituents as the growth medium plus 1.2% Difco agar and 25 mg of neutral red per liter. The sodium bicarbonate content of the medium of all cultures incubated in a 5% CO_2 atmosphere was 2.25 g/liter. The incubation temperature was 37 C.

Viruses. Rubella virus R-1, isolated from thyroid tissue culture of an infant with congenital rubella (21) and prepared in BHK_{21} cells (24), was assayed in BSC-1 cells by a previously published modification (20) of the hemadsorption-negative plaque test (10). Echovirus 11, Gregory strain, was grown in pGMK cells and assayed by production of cytopathic effect (CPE) in the same cells. Vesicular stomatitis virus (VSV), Indiana serotype, was grown in pGMK cells and assayed in BSC-1 cells by plaque formation, and

also in the other kinds of cells when used for interferon titration. Newcastle disease virus (NDV), California strain, was obtained as the allantoic fluid harvested 2 days after infection of 11-day-old chick embryos and was assayed by plaque formation on 2-day-old monolayers of chick embryo cells. Sendai virus, strain 52, was grown in a similar manner and assayed by production of hemadsorption in pGMK cells. Sindbis virus, strain Ar-339, was grown in chick embryo cells and assayed in a manner similar to NDV.

Production and assay of interferon. Except when mentioned otherwise, interferon was prepared by inoculating cell monolayers in 16-oz (80 cm^2) bottles with 1 plaque-forming unit (PFU) of NDV per cell contained in 30 ml of maintenance medium without serum. The fluids were harvested after 36 to 48 hr of incubation in a 5% CO_2 atmosphere. This procedure is similar to that described by Merigan et al. (14) for human foreskin interferon. Pooled fluids were acidified to *p*H 2.0 with HCl, stored 5 days at 4 C, brought to *p*H 7.0 with NaOH, subjected to low-speed centrifugation, and then centrifuged twice for 2 hr at 100,000 × *g*. The supernatant preparations were stored frozen at −20 C. Interferon assays were carried out on BSC-1 cell monolayers in petri dishes or on other cells where indicated. A 4-ml amount of test fluid diluted in maintenance medium was left in contact with the cells for 18 hr in a 5% CO_2 atmosphere. The cells were then washed once with medium without serum, or three times, in the case of interferon induced by Sindbis virus. Fifty to 80 PFU of VSV in 0.2 ml was allowed to adsorb for 1 hr, then agar overlay was applied. This procedure is similar to that described by Petralli et al. (18) for the assay of human interferon in foreskin cells. Plaques were counted after 2 days. The interferon titer is expressed as the highest twofold dilution giving at least 50% plaque reduction. The reciprocal of this value is the number of interferon units per ml. Two or three replicate plates were used for each dilution tested. Interferon activity was considered absent when, at a dilution of 1:8, there was less than 50% reduction in the number of VSV plaques.

RESULTS

Defectiveness of interferon production in Vero cells. The Vero cell line was selected for study as a possible candidate for a defective interferon system. The defective system was suspected because a small inoculum of rubella virus in pGMK fails to replicate to maximal titers [which has been explained (26) by the activity of endogenous interferon], whereas the virus grows to high titers in Vero cells regardless of the size of the inoculum (9). BSC-1 cells (rather than pGMK) were selected for control purposes, because it was found that the response of BSC-1 cells to interferon and their production of interferon was comparable to that of pGMK, and this cell line gave more consistent results than did different batches of pGMK cells. In addition, BSC-1 cells support the replication of rubella virus as demonstrated by interference to superinfection with other viruses (16, 20).

TABLE 1. *Induction of interferon in Vero, BSC-1, and human foreskin (FSK) cells with Newcastle disease virus*

Interferon source	Assay cells		
	Vero	BSC-1	FSK
Vero Expt 1	0[a]	0	0
	0	0	ND
2	0	0	0
	ND[b]	0	ND
BSC Expt 1	128[c]	512	512
	128	256	512
2	64	256	1,024
	128	512	256
3	128	256	512
FSK Expt 1	64	128	128
2	64	64	256

[a] <1:8.
[b] Not done.
[c] Units of interferon per milliliter.

The results of comparative attempts to produce interferon with NDV in Vero, BSC-1, and foreskin cells and to assay interferon in the same cells are given in Table 1. No interferon was detectable in fluid from Vero cultures at a 1:8 or higher dilution, whether tested on Vero, BSC-1, or human foreskin cells. BSC-1 cells were slightly better producers of interferon than foreskin cells, whereas foreskin cells were slightly more sensitive to interferon. Vero cells, however, were sensitive to interferon produced in BSC-1 or foreskin cells, but slightly less than BSC-1 cells. As expected (1, 6), no indication of species specificity between human and monkey interferon was found. Similar results were obtained in other experiments with BSC-1 and Vero cells. Five similar attempts to produce interferon in Vero cells failed to reveal activity at the lowest dilution tested (1:8 or 1:16). Differences in the capacity of VSV to replicate in the assay cells could not explain the results obtained. The average plaquing efficiency of the VSV stock was 0.8 in Vero cells and 0.3 in foreskin cells when compared to BSC-1 cells. Plaques could be conveniently counted in both Vero and BSC-1 cells after 36 hr, and 12 to 18 hr later in foreskin cells. No gross differences were observed between Vero and BSC-1 cells in the time at which plaques appeared and increased in number and size.

Development of NDV cytopathic effect was

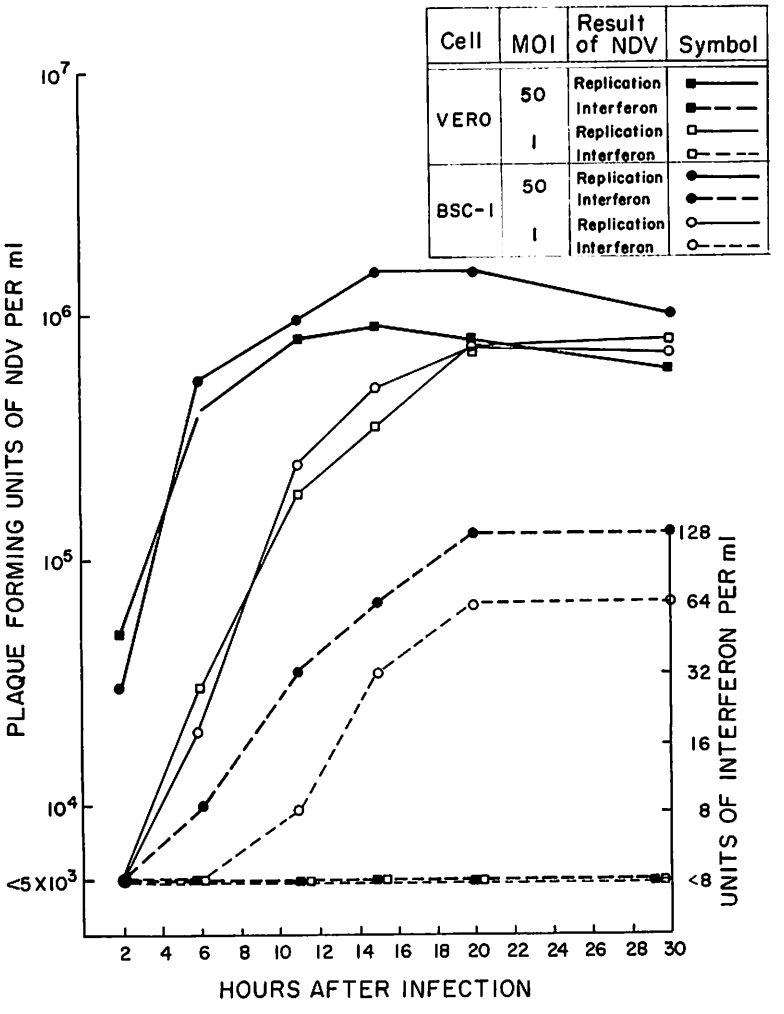

FIG. 1. *Single-cycle multiplication of Newcastle disease virus and production of interferon in Vero and BSC-1 cells, 200,000 per tube monolayer in 1 ml.*

somewhat more rapid in Vero cells than in the other cells. Interferon activity was therefore examined in fluids harvested 24 hr after infection of Vero cells rather than at 48 hr. Again, no activity was found. After challenge of Vero cells with NDV at a multiplicity of infection (MOI) of 20 and 0.05 rather than 1, or with ultraviolet-inactivated NDV (4), no interferon activity was found. In Fig. 1, the single-cycle multiplication of NDV and the appearance of interferon in tube cultures of Vero and BSC-1 cells are represented. Monolayers of 2×10^5 cells were infected at an MOI of 1 and 50 PFU of NDV in 0.2 ml; after 2 hr, they were washed five times, 1 ml of medium was added, and the cultures were harvested at different times of incubation. For each harvest, four replicate tubes were frozen and thawed three times, pooled, assayed for NDV, and also processed for assay of interferon. The replication of NDV in Vero and BSC-1 cells was remarkably similar; however, at no time was interferon found in Vero cultures. The inhibitor produced in BSC-1 cells was established as interferon by most of the criteria applied to human foreskin interferon (3), including insensitivity to NDV antiserum and absence of activity on chick cells. Foreskin and BSC-1 interferons were active on rabbit kidney cells (3), whereas comparable Vero fluids were not.

Although NDV is regarded as the most useful agent for producing interferon in high yield (25), other viruses reported to be satisfactory inducers

were tested. Sendai and Sindbis viruses were used under the same conditions as NDV. They produced more rapid CPE and fluids were therefore harvested after 24 hr. Acid inactivation of these viruses was limited to 2 days. No interferon activity was found in Vero fluids, whereas interferon titers of 1:512 and 1:64 were obtained in fluids of BSC-1 cells infected with Sendai virus, and of 1:32 and 1:16 in BSC-1 cells infected with Sindbis virus. Interferon was absent in fluids of Vero cells infected 2 or 4 days earlier with rubella virus at different MOI, but interferon was also not well demonstrable in similarly treated BSC-1 cells (titer 1:8 or, more frequently, < 1:8 on BSC-1 or foreskin cells).

The possibility was considered that Vero cells may produce interferon and simultaneously produce an interferon antagonist which is active in primate cells. Such a situation has been shown to exist between different types of murine cells (23). Different dilutions of interferon induced by NDV in BSC-1 cells were therefore made in undiluted Vero NDV-"interferon," and assayed on BSC-1 cells. It was found that the titer of the interferon induced in BSC-1 cells was normally expressed, indicating that an interferon antagonist was not produced by the Vero cell. It is concluded from the above experiments that Vero cells are defective in their ability to produce interferon; however, these cells are sensitive to exogenously added interferon.

Defectiveness of rubella virus interference in Vero cells. Rubella virus was titrated by the hemadsorption-negative plaque method in BSC-1 cells (20), by development of CPE in Vero cell tube cultures (9, 22), and by interference with the development of echovirus CPE in BSC-1 cells as originally described in pGMK cells (16). The titers obtained in repeat titrations by the three methods were similar ($10^{4.8}$ to $10^{5.2}$ infectious units per ml). This was predicted, since it has been shown that the hemadsorption-negative plaque titer (10), as well as the titer obtained by development of CPE in Vero cells (9), are similar to the titer of interference with echovirus CPE in pGMK cells. When Vero cells infected with rubella virus were challenged with 1,000 TCD_{50} of echovirus, there was complete destruction of the cell sheet which occurred rapidly and concurrently in all tubes, whether they had been inoculated 5 to 7 days earlier with low or with high dilutions of the rubella virus preparations. At the moment of challenge with echovirus, Vero cell monolayers containing rubella virus showed distinct but limited CPE when compared to uninoculated controls. The rubella virus CPE did not affect the continuity of the cell sheet nor did it interfere with the development of typical enterovirus CPE.

NDV. Attempts were made to demonstrate infection of Vero cells with rubella virus by the formation of hemadsorption-negative plaques upon challenge with NDV (20). Vero cell monolayers in petri dishes were inoculated with different dilutions of rubella virus, incubated under fluid medium, and challenged with NDV after 1, 2, 3, and 4 days. Since NDV hemadsorption was found to develop at about the same rate in noninfected Vero cells as in BSC-1 (20) and in pGMK (10) cells, erythrocytes were added at the same time as to the other cell types, i.e., after 15 to 18 hr. No hemadsorption-negative plaques or inhibition of NDV hemadsorption was seen in Vero cells infected with rubella virus. Sheep erythrocytes were adsorbed to Vero cell monolayers whether or not they were infected with rubella virus and without regard to the size of the rubella virus inoculum or of the time they had been infected. Treatment with NDV antiserum after adsorption of NDV, designed to avoid spurious binding of erythrocytes (10), did not result in the appearance of plaques. Hemadsorption-negative plaques developed in control cells, which included pGMK, BSC-1, human embryonic fibroblasts, and three strains of human foreskin cells, and in CV-1 cells, another stable line of African green monkey kidney origin.

The above results with NDV and echovirus suggested that challenge virus may multiply in single Vero cells which were coinfected with rubella virus. However, an alternative explanation would be that relatively few Vero cells were infected with rubella virus at any time and with any inoculum. The following experiments were performed in Vero cells to establish and to measure maximal infection with rubella virus.

Vero cell monolayers were infected with rubella virus at an MOI of 0.1 to 1. After 4 days of incubation, part of the cultures was infected with NDV at an MOI of 10 and further processed for the single-cell hemadsorption test, as described by Marcus and Carver (11). The percentage of cells hemadsorbing erythrocytes was estimated by counting 200 cells. The other part of the cultures was used to establish the percentage of cells infected with rubella virus by infectious center assay (21; Rawls, Desmyter and Melnick, *in press*). The results are given in Table 2. Essentially all Vero cells under the given conditions were apparently infected with rubella virus, and virtually all such cells allowed development of NDV hemadsorption, thus proving coinfection of individual Vero cells with rubella virus and NDV. The development of NDV hemadsorption was measured by the single-cell technique in Vero and BSC-1 cells; some were coinfected with rubella virus under conditions of maximal infection and some were not (Table 3). Vero cells

TABLE 2. *Coinfection of Vero cells with rubella virus and Newcastle disease virus*[a]

Expt no.	Cells with NDV hemadsorption (%)	Determination of rubella infection of Vero cells		
		Estimated cells per tube	Tubes with rubella/ tubes tested	InD_{50} (cells)[b]
1	97	3[c]	4/5	
		1	4/5	0.8
		0.3	0/4	
2	98	3	5/5	
		1	2/5	1.3
		0.3	0/3	
3	98	3	4/5	
		1	3/5	0.9
		0.3	1/5	

[a] Vero cells were infected with rubella virus and incubated 4 days at 37 C. The cultures were superinfected with NDV and the per cent of hemadsorbing cells was determined after 15 to 18 hr of additional incubation. Replicate cultures were used to determine the per cent of cells infected with rubella virus by infectious center assay.

[b] Inhibitory $dose_{50}$ calculated by method of Reed and Muench.

[c] When tubes contained 10 and 100 cells, four of four tubes tested were found to contain rubella virus in all experiments.

produced NDV hemadsorption whether or not they were infected with rubella virus; in BSC-1 cells, NDV hemadsorption was markedly inhibited in the coinfected cells. Vero and BSC-1 cells which were not infected with NDV did not adsorb sheep erythrocytes.

VSV. The interference of rubella virus with VSV was determined by examining virus replication and plaque formation in Vero and BSC-1 cells coinfected with rubella virus. Vero and BSC-1 cells maximally infected with rubella virus and control cells were infected at a high and low MOI of VSV and washed after 2 hr of adsorption. The yields of VSV after 36 hr of incubation of the cultures are given in Table 4. By infectious center assay, it was determined that over 90% of the BSC-1 and Vero cells were infected with rubella virus, yet the replication of VSV in Vero cells infected with rubella virus was more than a 1,000-fold greater than in BSC-1 cells infected with rubella virus. When a VSV preparation was titered in monolayers of the same sets of cells, a titer of 1.3×10^7 PFU/ml was obtained in noninfected Vero cells and 1.0×10^7 PFU/ml in Vero cells infected with rubella virus. The plaque characteristics were comparable in both infected and noninfected monolayers. In contrast, a titer of 2.7×10^7 PFU/ml was obtained in noninfected BSC-1 cells, whereas no plaques (less than 500 PFU/ml) appeared in the rubella-infected BSC-1 cells.

The experiments with echovirus, NDV, and VSV demonstrated that rubella virus interference is defective in Vero cells. This defectiveness appeared to operate at the level of the single infected cell. Neither the presence of rubella virus synthesis nor the ultimate, if delayed, cytolytic effect of rubella virus in the Vero cell seemed to interfere with the replication of challenge virus.

DISCUSSION

Although cells which are defective in interferon production or are insensitive to interferon may have considerable potential in the elucidation of viral functions related to interferon, no intensive search for such cells has been made in recent years. Results of earlier studies concerning the relative efficiency of production and response to interferon by various types of cells have been summarized by Ho (7). The described defectiveness in interferon production upon viral challenge of Vero cells is the first instance in which monkey

TABLE 3. *Development of NDV hemadsorption in Vero and BSC-1 cells infected or not infected with rubella virus*[a]

Expt no.	Vero cells		BSC-1 cells	
	With rubella	Without rubella	With rubella	Without rubella
1	97[b]	99	1.5	100
2	98	100	0	98

[a] Vero and BSC-1 cells were infected with rubella virus and incubated 4 days; then they were superinfected with NDV and examined for the number of cells which developed hemadsorption.

[b] Percentage of cells in which NDV induced hemadsorption of sheep erythrocytes.

TABLE 4. *Rubella virus interference with multiplication of vesicular stomatitis virus in Vero and BSC-1 cells*

Cell types	Infected with rubella virus[a]	VSV (MOI 0.001)	VSV (MOI 20)
Vero cells.....	−	1.3×10^{7}[b]	1.5×10^7
Vero cells.....	+	1.0×10^7	0.3×10^7
BSC-1 cells....	−	2.7×10^7	1.0×10^7
BSC-1 cells....	+	$<5 \times 10^2$	1.0×10^3

[a] Cells were infected with rubella virus and incubated 4 days; then they were superinfected with VSV and harvested after 36 hr.

[b] Number of PFU obtained per 200,000 cells grown in 1 ml of medium.

cells seem to be qualitatively defective in this function. Obviously, the data presented do not rule out the genetic capability of Vero cells to code for interferon (2), nor has the production of interferon in amounts too small for detection been excluded. However, the data strongly suggest loss of, or interference with, the transcription or translation of the genetic information which codes for interferon production. The sensitivity of Vero cells to exogenous interferon supports the concept that the afferent and efferent pathways of the interferon system are controlled by different genetic loci.

Rubella virus is unusual among human viruses in its lack of production of CPE coupled with interference with the replication of a large number of unrelated viruses. It would seem that a single intracellular mechanism is responsible for such interference in different cells and with different viruses. Rubella virus interference may be due to any of the following mechanisms. (i) It may be conveyed by the virus genome, the host cell genome playing no part. Studies of rubella virus interference with NDV replication in pGMK cells using dactinomycin (10, 11), an inhibitor which essentially blocks DNA transcription, supports the conclusion that such a mechanism is indeed operative in this case. In Vero cells, however, NDV hemadsorption develops equally well whether or not the cells are coinfected with rubella virus. The data indicate that the presence of replicating rubella virus does not in itself exclude the replication of NDV and that rubella virus interference is not solely a function of the rubella virus genome. The loss of rubella virus interference to NDV replication in pGMK cells, when the rubella-infected cells were dispersed by trypsinization (11), is compatible with this conclusion. (ii) The interference could be mediated by the host cell genome in a manner unrelated to interferon. (iii) The interference could be mediated by the host cell genome through the interferon system; that is, rubella virus may convey interference by the production of interferon or by inducing any other step in the interferon pathway leading to the production of translation-inhibitory protein (12). Studies of rubella virus interference with VSV and poliovirus in pGMK cells combined with the use of dactinomycin (26) have led to the conclusion that rubella virus interference in most, if not in all, instances is mediated by interferon. However, the data upon which this conclusion was drawn are compatible with any mechanism involving transcription of the host cell genome, and the interferon system could either provide the sole mechanism of interference or act as an auxiliary mechanism. The simultaneous occurrence of the two unusual characteristics of defectiveness in interferon production and absence of rubella virus interference in Vero cells is highly suggestive of a causal relationship between the two properties. A definite clarification of the problem awaits tools to study production, fate, and activity of interferon at the level of the same cell. The present data, however, bring support to the hypothesis that rubella virus interference is mediated through the interferon pathway and at the initial step of interferon production.

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Induction of Interferon in Hybrid Clones: Vero 153–Mouse Myeloma and Vero 153–Human Lymphocyte Cells

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Abstract—*A Vero cell line (Vero 153) resistant to 8-azaguanine and unresponsive to viral induction of interferon was isolated. This primate (African green) cell line was fused with mouse myeloma (S194/5) and normal human lymphocytes from peripheral blood. All Vero 153–mouse hybrids, 8 primary and 12 secondary clones, produced virus-induced mouse but not primate interferon. This occurred even in cultures where >90% of primate chromosomes were retained. Similarly 7 primary and 3 secondary Vero 153–human clones synthesized virus-induced interferon. This could be neutralized by anti-human fibroblast (β) but not by anti-human leukocyte (α) interferon antisera. The unresponsive nature of Vero 153 cells to interferon induction by viruses was not changed by the presence of interferon producing genomes from other cells. However, despite the inability to produce interferon, the Vero cell was able to play a role in the determination of the type of interferon made in the hybrid cell.*

INTRODUCTION

Somatic cell hybrids have been used previously in the study of the interferon production mechanism. These studies have shown that in hybrid cells the interferon genes of both interferon-producing cells may remain functional (1), or if one were a nonproducer, the interferon genes could be activated by their interferon-producing partners (2, 3) or conversely may remain in the suppressed state (4).

Vero, a continuous line derived from the African green monkey kidney (5), was the first cell type shown to be incapable of producing interferon when challenged with a variety of inducing agents (6). Recently we isolated a clone

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of Vero cells which was resistant to the nucleic acid base analog 8-azaguanine. This mutant, designated Vero 153, retained the unresponsive interferon production system of its parent. By fusing Vero 153 with a bromodeoxyuridine (BrdU) resistant mouse myeloma cell line and with normal human lymphocytes, we are able to isolate (using HAT medium) 20 Vero 153–mouse and 10 Vero 153–human hybrid clones. In this paper the data on the induction of interferon in these hybrids are presented and the implications of the results discussed.

MATERIALS AND METHODS

Cells and Culture Conditions. Unless otherwise specified, all culture medium was made up of Eagles' minimal essential medium (MEM) supplemented with 10% Mycoplasma-free fetal calf serum (FCS) (Flow Laboratories), 100 units/ml penicillin and 100 μg/ml streptomycin.

Vero 153, a hypoxanthine guanine phosphoribosyltransferase (HGPRT) -deficient mutant, was derived from Vero cells by stepwise selection in 8-azaguanine-containing medium.

Mouse myeloma cell line (S194/5) obtained from the Salk Institute, California, was grown in culture medium containing 10^{-4} M bromodeoxyuridine (BrdU). BSC-1 and L-929 cultures were carried continuously in our laboratory. These lines were originally obtained from the American Type Culture Collection.

Human lymphocytes were isolated from heparinized whole blood by Ficoll-Hypaque gradient centrifugation (7).

Hybridization. Polyethylene glycol (PEG 1000, Sigmal Chemical Co.) 35% (v/v) was used as the fusion agent. Immediately prior to fusion, Vero 153 monolayer cultures were trypsinized and washed free of serum. The 10^7 Vero 153 cells were then mixed with 10^8 mouse myeloma or human lymphocytes in serum-free suspension MEM. The fusion procedure was carried out as described by Gefter and coworkers (8).

The mixed cell population, after PEG treatment, was seeded in 120-mm T flasks in 50 ml culture medium and incubated at 37°C. Forty-eight hours later the culture was trypsinized and the cells redistributed to 60-mm dishes at a density of approximately 2×10^6 Vero 153 cells/dish in (hypoxanthine, aminopterin, thymidine) HAT medium (Flow Laboratories) supplemented with 10% fetal calf serum and 0.3% L-glutamine. The cultures were henceforth fed twice weekly. In general, hybrid clones appeared 12–20 days later. Subcloning of hybrid cells was carried out by plating approximately 10^2 cells per 35-mm dish or 1–2 cells per microwell in Linbro 96-well plates.

Testing for Interferon Production. Monolayer cultures containing about 1.5×10^6 cells per 16-mm well (Costar's 24-well plate) were infected with

UV-inactivated or live Newcastle disease virus (NDV, strain California) at a virus input of 0.1–10 plaque-forming units/cell. After 90 min adsorption, the inoculum was removed and the cultures washed and reincubated with 0.5 ml MEM. Eighteen to 24 hours later, the supernatant was collected and acidified, and assayed for interferon activity four days later (9). The presence of interferon was assessed by the plaque-reduction method in mouse L-929 or monkey BSC-1 kidney cell monolayers for mouse or primate interferon, respectively, using vesicular stomatitis virus (VSV) as the challenge virus (9). The BSC-1 cells are comparable to human diploid foreskin fibroblast in sensitivity to human IFN-α and IFN-β. In our BSC-1 cells, one unit of human IFN-α reference reagent G093-901-527 and human IFN-β reference reagent G023-902-527 registered as 1.1 and 1.3 units of antiviral activity, respectively. The results in this study are expressed as NIH reference units. The stanard reagents were kindly provided by Drs. J. K. D. Dunnick and M. W. Myers of NIAID Antiviral Substances Program.

Interferon Characterization. Rabbit antisera against human fibroblast (IFN-β) or human leukocyte (IFN-α) interferon were similarly obtained from the NIAID Antiviral Substances Program. Samples of the harvested fluid were incubated for 1 h at room temperature with appropriate dilutions of the anti Hu-IFN-α or Hu-IFN-β globulins before being assayed for residual antiviral activity.

Chromosome Analysis. Hybrid cells prepared for cytogenetic studies were sampled at the same passage level as those used for testing interferon production. Approximately 1×10^6 cells were seeded in 60-mm dishes. After 48 h, colcemid was added (1.5 μg/ml) and the cells harvested 3–4 h later. They were placed in hypotonic 0.04 M KCl for 25 min and the cells then fixed with 3:1 methanol–acetic acid. Fixed cells were spread on wet slides and allowed to air dry. A 10% Giemsa solution was used as chromosomal stain.

Table 1. Interferon Induction with UV-NDV/NDV

Cell type	Interferon titer (NIH U/0.5 ml), induction by	
	UV-NDV[b]	NDV
AGMK[a]	<4	>108
Vero	<4	<4
Vero 153	<4	<4
Mouse myeloma (S194/5)	<4	205
Human lymphocytes	108	107
Vero 153–mouse myeloma (summary all clones)	<4	28–180
Vero 153–human lymphocyte (summary all clones)	<4	9–91

[a] Primary African green monkey kidney cells.
[b] Ultraviolet inactivated Newcastle disease virus of equivalent titer to NDV prior to inactivation.

The trypsin–Leishman technique was employed for G-band induction (10). Chromosomes were photographed with a Zeiss photomicroscope using a 40 × 1.25 Numerical Aperture (NA) or 100 × 1.25 NA oil planachromatic with bright field illumination.

RESULTS

A comparison of the interferon productivity by different cell types in response to infection with NDV or UV-irradiated NDV is summarized in Table 1. Positive interferon response was observed in mouse myeloma (S194/5), human lymphocyte, and primary African green monkey kidney (AGMK) cells when NDV was used as the inducer. UV-irradiated NDV was capable of eliciting interferon activity in human lymphocytes only. No induction was detected in Vero 153 with either inducer.

The results of interferon production by Vero 153–mouse hybrids are presented in Table 2. All 20 clones (eight primary and 12 secondary) from two

Table 2. Distribution of Interferon Production among Vero 153–Mouse Myeloma Hybrids Induced with NDV[a]

Experimental series	Hybrid clone[b]	Interferon titer NIH U/0.5 ml	
		Primate[c]	Mouse[d]
I	1	<4	180
	1a	<4	159
	1b	<4	177
	1c	<4	28
	2	<4	120
	2a	<4	54
	3	<4	31
	4	<4	110
	5	<4	108
	6	<4	108
II	7	<4	102
	7a	<4	36
	7b	<4	11
	7c	<4	17
	7d	<4	54
	7e	<4	66
	7f	<4	76
	8	<4	>108
	8a	<4	37
	8b	<4	39

[a]Induction by UV-irradiated NDV was negative for both primate and mouse interferon in all clones.
[b]Arabic number denotes primary clone; numbers with letters indicate corresponding subclone.
[c]Titered in BSC-1 monkey kidney cells.
[d]Titered in L-929 mouse fibroblasts.

Table 3. Distribution of Interferon Production among Vero 153–Human Lymphocyte Hybrids Induced with NDV[a] and Antibody Characterization

	Hybrid clone[b]	Interferon titer (NIH U/0.5 ml)	Residual activity (NIH U/0.5 ml)	
			Hu-IFN-α	Hu-IFN-β[c]
Experimental series I	1	91	100	<1
	1a	55	55	<1
	1b	23	20	<1
	2	30	35	<1
	3	89	89	<1
	4	17	20	<1
	5	37	37	<1
	5a	12	12	<1
Experimental series II	6	10	10	<1
	7	9	10	<1
Control Hu-IFN-α (NDV-induced) (9)		100	<1	100
Control Hu-IFN-β (PolyrI:rC-induced) (7)		100	100	<1
Control AGMK-IFN (NDV-induced)		100	100	100

[a] Interferon response was negative in all hybrid cultures induced with UV-irradiated NDV.
[b] Arabic numbers denote primary clones; numbers and letters indicate corresponding subclone.
[c] Anti-Hu-IFN-α and anti-Hu-IFN-β were used at a final concentration of 1:100 and 1:20, respectively.

separate fusion experiments were found to produce mouse interferon. In contrast, primary interferon activity was not detected in any of the hybrid cultures.

Similarly, all 10 Vero 153–human hybrid clones (seven primary and three secondary) produced interferon on induction. Interestingly, the activity of this interferon was neutralized by rabbit anti-human fibroblast but not by anti-human leukocyte interferon sera (Table 3).

As indicated in Tables 2 and 3, UV-NDV failed to induce interferon in all hybrid cells under investigation. It was active only in human peripheral lymphocyte cultures (Table 1).

Routinely stained metaphase plates from a mouse myeloma and a Vero 153 cell are presented in Figs. 1 and 2, respectively. The mouse chromosomes were either acrocentric or acentric. The majority of the monkey chromosomes (approximately 80%) were biarmed and were thereby readily distinguishable from the mouse chromosomes. Twenty metaphase spreads from each Vero 153–mouse hybrid clone were analyzed. Results of the analysis were recorded in Table 4. A large variation in the number of chromosomes per cell was observed within the same culture. As can be seen from Figs. 3 and 4, the loss of

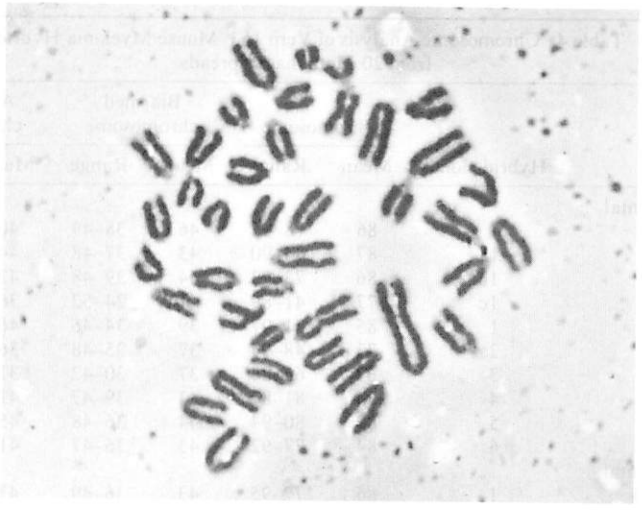


Fig. 1. Metaphase plate from a mouse myeloma (S194/5) cell.

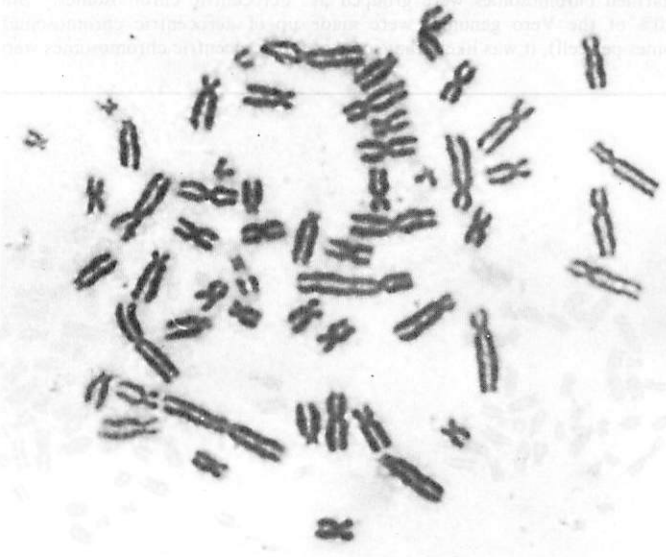
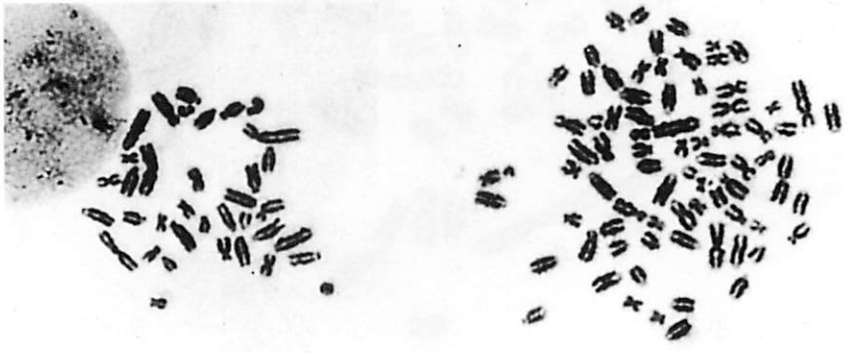


Fig. 2. Metaphase plate from a Vero 153 cell.

Table 4. Chromosome Analysis of Vero 153–Mouse Myeloma Hybrids from 20 Metaphase Spreads

		Chromosome		Biarmed chromosome		Acrocentric chromosome[a]	
	Hybrid clone	Mean	Range	Mean	Range	Mean	Range
Experimental series I	1	86	78–93	46	38–49	40	37–49
	1a	87	84–90	43	37–48	44	38–48
	1b	86	73–93	44	39–48	42	34–51
	1c	77	41–89	41	24–52	36	17–41
	1	85	71–97	39	34–46	46	35–54
	2a	73	48–94	37	25–48	36	22–46
	3	69	62–84	37	30–42	32	31–48
	4	84	81–87	43	39–47	41	38–47
	5	89	80–94	44	36–48	45	39–50
	6	84	77–92	43	36–47	41	36–46
Experimental series II	1	86	77–95	43	36–49	43	38–47
	1a	69	39–85	37	23–50	32	16–39
	1b	70	48–90	49	21–52	21	18–40
	1c	74	47–91	40	20–51	34	23–40
	1d	66	32–83	38	17–40	28	15–43
	1e	72	38–81	39	18–48	33	20–37
	1f	75	39–88	41	22–50	34	17–39
	2	89	77–96	45	39–49	44	38–47
	2a	68	36–84	37	12–44	31	24–42
	2b	72	41–87	45	22–45	27	19–42

[a]All nonbiarmed chromosomes were grouped as "acrocentric chromosomes." Since approximately 20% of the Vero genomes were made up of acrocentric chromosomes (about 12 chromosomes per cell), it was likely that some of the acrocentric chromosomes were of monkey origin.



Figs. 3, 4. Metaphase plates from Vero 153–mouse myeloma hybrid cells.

Fig. 5. A G-banded Vero 153 cell. Arrow indicates the Vero chromosome having similar band pattern to a particular human chromosome the number of which is circled.

chromosomes appeared to be random, that is, not confined to one species alone.

A G-banded Vero 153 metaphase plate is presented in Fig. 5. With the exception of five chromosomes, the rest of the monkey complement could be differentiated from the human chromosomes. Ten banded metaphase spreads from each Vero–human hybrid culture were analyzed. High ploidy (>120) was observed in all 10 hybrid clones; most chromosomes conformed to the Vero band pattern (see Table 5). Example of a Vero–human chromosome spread is presented in Fig. 6.

DISCUSSION

Our hybridization experiments showed that the Vero genome remained nonresponsive to interferon induction when functional mouse or human interferon genes resided in the same cell. Twenty-one Vero–mouse L-cell hybrid clones constructed earlier by Emeny and Morgan (11) also gave no evidence that Vero (monkey) interferon could be induced in these cells. However, chromosome analysis was performed in only one clone only, and the reason for such nonresponsiveness could be ambiguous since unilateral loss of primate chromosomes from primate–rodent hybrids is not uncommon. The

fact that only the rodent form of lactate dehydrogenase and glucose-6-phosphate dehydrogenase enzymes were detected in the hybrid clones and the fact that a majority of the hybrids were only sensitive to mouse interferon indicated that in the Vero–mouse L-cell system the simian chromosomes were lost preferentially. More convincing data of selective chromosome loss was found when cytogenetic studies were performed in one of the clones. This revealed the presence of only two small metacentric chromosomes, typical of primate origin; the rest of the hybrid complement was telocentric. In view of such massive loss of the Vero genome, it was debatable whether any Vero interferon gene(s), if such genes exist, had been retained.

Unlike the Vero–mouse L-cell system (11), unilateral loss of the monkey chromosomes was not observed in the hybrid clones which we constructed. Cytogenetic data showed that 70–90% of the simian genome was retained in the Vero–mouse myeloma hybrids, and almost twice the number of the average Vero chromosome constitution (modal Vero 153 chromosome number was 56–57) was found in the Vero–human lymphocyte cultures. We deduced with confidence, therefore, that the failure of these hybrids to produce monkey

Fig. 6. A G-banded metaphase plate from a Vero 153–human hybrid cell.

interferon was not the result of hybridization artifacts but were rather an inherent characteristic of the Vero genome which could not be influenced by the presence of induced interferon producing genomes from other cells.

Deletion or mutation of the interferon gene, within either the structural or "recognition" sites, had been suggested to be the cause of the Vero's inability to produce interferon (11). Although this speculation offered an attractive explanation of our present findings, circumstances do exist where inactive but otherwise nondefective interferon genes remained inoperative when hybridized with producive fusion partners (4).

In our study considerably less interferon was produced by eight of the 10 Vero–human hybrid clones than would be produced on a per cell basis by the human parental cells. The same occurred in 50% of the Vero–mouse clones. This observation could mean that the control mechanisms which resulted in a negative interferon response in Vero cells had also partially affected the human or mouse genome in the hybrid. Competition between Vero and human or mouse repressor molecules could result in a reduced production of interferon of the human or mouse species. If this were the case, however, this should also result in partial release of a negative control of Vero interferon production. On the contrary, monkey interferon was never detected in any of the hybrid clones. The reduction of human and mouse interferon yields by our hybrid cells was therefore not likely to be the consequence of the negative effect exerted by the Vero genome.

Alternatively, a decrease in the proportion of cells in a hybrid culture which retained the human or mouse interferon gene could be responsible for the reduction of the respective interferon yield by that culture. Results from cytogenetic studies seemed to lend support to this suggestion. A large variation in the number of chromosomes per cell was observed within the same culture in both the Vero–mouse and the Vero–human systems. As a general observation, hybrid clones with fewer average numbers of non-Vero chromosomes and/or greater heterogeneity tended to be those with a low interferon productivity following induction. In the Vero–human hybrids homologous chromosomes were often found to be present or absent concomitantly. This observation indicated that the portion of the human genome being retained in the hybrids was less representative of the whole karyotype than would be the case if each of the chromosomes that were retained belonged to a different linkage group.

The concomitant presence or absence of homologous chromosomes also suggested that endoreduplication could be one of the reasons for the high ploidy found in the Vero–human hybrid cultures.

Results presented in Table 1 show that both mouse myeloma cells and normal AGMK, the original source of Vero cells, could not be induced by UV-irratiated NDV to synthesize interferon. It came as no surprise, therefore,

that all Vero–mouse hybrids were nonresponsive to UV-irradiated NDV induction. What was unexpected, however, was the finding that hybrids between human lymphocytes which could be induced to make interferon by UV-irradiated NDV and Vero had also become nonresponsive to UV-irradiated NDV induction. Whether this means the hybrid cells have the membrane characteristic of the Vero (AGMK) partner or whether other internal facets of induction are of the Vero (AGMK) sort cannot be decided by these experiments.

The dominance of Vero cell phenotype over that of the human lymphocytes was also demonstrated in the control of the type of interferon being synthesized. Although human lymphocytes have the ability to produce both α (leukocyte) and β (fibroblast) interferon (12, 13), when induced by NDV they respond with α synthesis. The hybrids, however, produced human fibroblast (β) rather than α interferon, a not uncommon observation with hybrids of leukocytes and fibroblasts (14). Thus, despite the inability to produce interferon, the Vero genome seemed to play an active role in the determination of the type of interferon induction in the hybrid system.

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VERO Cells in Rickettsiology

Introduction

Rickettsiae are small (399×600 nm), pleomorphic, Gram negative coccobacilli and are known to be the etiological agents for epidemic typhus, spotted fever, and tsutsugamushi disease.

Except for *Rochalimaea quintant*, all rickettsiae multiply only in living cells. Accordingly, embryonated eggs and experimental animals have been used for research of rickettsiae for a long time. Recently, tissue cultures have become available for the isolation and identification of rickettsiae. Cells derived from chickens, mice, monkeys, and humans have been used. When rickettsiae multiply in cultured cells, they produce a different types of cytopathologic change from that of typical CPE produced by viral infections. Specifically infected cells develop both granules and vacuoles and rickettsiae can be observed through various staining methods.

Vero cells permit multiplication of many types of rickettsiae. In addition, it has been reported that three rickettsiae of the spotted fever group produce plaques in Vero cells in a manner similar to viral plaque production (p. 151). Isolation of spotted-fever-group rickettsiae is usually conducted by inoculation of blood specimens into guinea pigs. However, recently, Vero cells have been used for isolation of some rickettsiae directly from patients' specimens (p. 156). Also, fixed Vero cells infected by rickettsiae have been used for detection of patient's antibodies using an immunofluorescent antibody technique.

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Plaque Assay of Rickettsiae in a Mammalian Cell Line

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Clear-cut and repeatable plaque assays were obtained for three rickettsiae of the spotted fever group (*Rickettsia rickettsi*, *R. conori*, and *R. montana*) in Vero cells used in a manner similar to that for arboviruses. In addition, three typhus group agents (*R. typhi*, *R. canada*, *R. prowazeki*) induced plaques in these cells. In preliminary tests *Coxiella burneti* (Nine Mile strain) failed to produce plaques. Comparable results were obtained in plastic flasks and plastic culture trays incubated in ambient air with or without addition of *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfinic acid buffer. Larger and more well defined *R. rickettsi* plaques were produced when cultures were overlaid with Leibovitz (L15) medium than with either medium 199 or Eagle medium. Phosphate-buffered saline containing bovine plasma albumin (fraction V), in contrast to brain heart infusion broth, as a diluent for preparing inocula consistently permitted development of larger and more numerous plaques with three agents: *R. rickettsi*, *R. conori, and R. montana.* When *R. rickettsi* and *R. typhi* were assayed in parallel in primary chicken embryo cultures and Vero cells, comparable results were obtained, but with *R. canada* results in Vero cells were superior. In contrast, *R. prowazeki* produced inconsistent results in Vero cells.

Methods for plaque assays of rickettsiae in primary monolayer cultures of chicken embryo cells were described by Kordova (2), McDade et al. (3), Weinberg et al. (4), and Wike et al. (6). Weinberg also reported plaque production by *Rickettsia rickettsi* in monolayer cultures of a monkey-kidney cell line (Vero cells). To our knowledge, no further investigations of rickettsial plaquing in established cell lines have been reported.

We describe here improved methods for plaquing six pathogenic rickettsiae in Vero cell monolayers, and report effects of various diluents, buffers, incubation temperatures, and overlay media on plaque size and titer.

MATERIALS AND METHODS

Rickettsial seeds. Sources of the organisms tested were infected yolk sacs of embryonated egg passage. Two had undergone prior passages in guinea pigs.

Agents tested were *R. rickettsi* "R" strain (53EP), *R. conori* Simko strain (13EP), *R. montana* M/5-6B strain (20EP), *R. typhi* Wilmington strain (112EP/15GP/1EP), *R. prowazeki* ZRS strain (15GP/2EP) and (4EP), *R. prowazeki* Breinl strain (15GP/2EP), *R. canada* (8EP), and *Coxiella burneti* Nine Mile strain Phase I (306GP/2EP).

Cells. The Vero cell line derived from African green monkey kidney by Yasamura and Kawakata (7) was obtained in its 122nd passage from the American Type Culture Collection, Rockville, Md. It was used in its 135th to 145th passage.

Culture vessels. Plaque assay culture vessels were 30-ml plastic flasks (Falcon Plastics, Los Angeles, Calif.), or plastic trays (model FB16-24TC) containing 24 wells with adhesive plastic-sheet tray covers from Linbro Chemical Co., New Haven, Conn.

Media and additives. All cell culture media were obtained in powdered form from Grand Island Biological Co., Grand Island, N.Y., and prepared according to manufacturer's instructions. Both growth and overlay media were used at pH 7.4.

Stock cell cultures were grown in medium 199 containing Earle salts to which had been added 5% fetal bovine serum and 10% tryptose phosphate broth (Difco, Detroit, Mich.) plus 100 units of sodium penicillin G and 100 μg of streptomycin sulfate per ml. The serum, obtained from Microbiological Associates, Inc., Bethesda, Md., was inactivated for 1 h at 56 C.

Primary overlay media were medium 199, Leibovitz medium (L15), and Eagle minimum essential medium (MEM) containing 5% fetal bovine serum, 10% tryptose phosphate broth, and 1% agarose (Seakem, Marine Colloids, Inc., Rockland, Me.). Secondary overlay media differed from these only by the addition of 1 ml of 1% neutral red dye per 100 ml of overlay medium (1:10,000 final concentration neutral red).

Effects of the addition of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (HEPES; Calbiochem, San Diego, Calif.) to overlay media was tested

in Vero cell cultures in flasks and trays. Parallel titrations of *R. rickettsi* were performed in each type vessel, with and without 10 mM HEPES buffer, in each of the overlay media. Cultures were incubated in ambient air at 32 C for 7 days before receiving the second overlay.

Dilutions of rickettsial seeds were made in either 0.15 M phosphate-buffered saline (PBS) containing 0.75% bovine plasma albumin (BPA) fraction V (Armour Pharmaceutical Co., Kankakee, Ill.) or brain heart infusion broth (BHI, Difco) as prepared by Wike et al. (5). Both were used at pH 7.0 and at 0 C.

Cell cultivation. Methods used for maintenance of Vero cell stock cultures and preparation of cultures for plaque assays were as described by Earley et al. (1), but modified for use of 30-ml plastic flasks or 24-well plastic trays. Flasks were planted with 5 ml of a cell suspension containing 5×10^5 cells per ml; wells of trays received 1 ml of this suspension. Monolayers were used for plaque assay after 48 h of incubation at 37 C.

Plaquing procedures. Inoculations of plaque assay cultures were performed with a precision pipetting system (Medical Laboratory Automation, Inc., Mount Vernon, N.Y.). Flasks from which medium had been decanted were inoculated with 0.1 ml, and wells of trays received 0.05 ml of inoculum. Inocula were allowed to adsorb for 1 h at room temperature on a mechanical rocking platform. Then, 5 ml of the appropriate overlay medium equilibrated to 44 C was applied to monolayers in flasks and 1 ml was applied to those in tray wells. After incubation at a temperature and time found appropriate for the particular rickettsia, 2 ml per flask or 0.5 ml per tray well of secondary overlay was applied in subdued room light. Vessels were covered with aluminum foil and reincubated. Plaques, often visible after 4 h, were usually read after overnight incubation. Incubation temperatures after primary overlay were 27, 32, 35, or 37 C. Secondary overlays were usually applied after 5 or 6 days of incubation, but various intervals up to 20 days were sometimes used depending on the development of satisfactory plaques.

Comparative titrations of four rickettsiae were done in Vero cell monolayers and in primary cultures of chicken embryo (PCE) as described by Wike et al. (6).

RESULTS

Effects of incubation time and temperature. In all cases, the sizes of plaques in Vero cell cultures reflected time and temperature of incubation (Fig. 1). Spotted fever group organisms caused plaques 1.0 to 2.0 mm in diameter within the 32 to 35 C range, providing that the secondary overlay was applied at a satisfactory time. Distinct plaques were produced by *R. rickettsi* when stained with secondary overlay after 5 days of incubation at 37 C (1.0 mm), by *R. montana* after 6 days at 35 C (1.5 mm), and by *R. conori* after 6 days at 35 C (2 mm). *R. prowazeki* ZRS strain (4EP) repeatedly produced well-defined plaques of 1.0 mm diameter after 6 to 8 days at 37 C. However, another seed of ZRS strain (15GP/2EP) as well as of the Breinl strain (15GP/2EP) only occasionally formed plaques; these were less than 1 mm in diameter, poorly defined, and visible only after 20 days of incubation at 32 C. *R. canada* plaques, which varied from 1 to 2 mm in diameter, were not well defined until 12 days after incubation at 37 C (6 days after secondary overlay had been applied). *R. typhi* formed plaques of 1.0-mm diameter only after 20 days of incubation at 27 C. *C. burneti* (Nine Mile strain), tested at 32, 35, and 37 C for 14 days and at 27 C for 20 days, did not form plaques.

Effects of overlay media and modifications. In parallel assays of *R. rickettsi* performed in flasks and trays incubated at 32 C for 7 days in ambient atmosphere, only moderately well-defined plaques, about 1.0 mm or less in diameter, were produced in cultures that received medium 199 or MEM overlay medium (Fig. 2). Cultures that received L15 overlay medium produced well-defined plaques 2.0 mm in diameter. HEPES buffer had no effect on plaque development.

Effects of diluents on plaque size and titer. Parallel plaque assays were performed in Vero cell cultures to compare PBS + BPA with BHI as a vehicle for dilution of seeds of *R. rickettsi*, *R. montana*, and *R. conori*. Various incubation times and temperatures were used. Plaques of *R. rickettsi* obtained through use of PBS + BPA were larger (1.5 to 2.0 mm diameter) than those resulting from use of BHI (1.0 to 1.5 mm diameter) (Table 1). Titers of *R. rickettsi* seeds in which PBS + BPA was used were equal to or higher than (up to 0.53 dex) those obtained with BHI. Results with *R. montana* were similar. *R. conori* plaques were 2.0 mm in diameter regardless of diluent used; the titer was only 0.20 dex higher when diluted in PBS + BPA. Although plaque values did not differ greatly, titers were consistently higher and plaques larger when PBS + BPA was used as diluent.

Rickettsial titers compared in Vero and PCE cultures. Parallel plaque assays were performed in Vero and PCE cultures with identical inocula of *R. rickettsi*, *R. typhi*, *R. canada*, and *R. prowazeki* (15GP/2EP). All tests were performed with two or four replicate culture vessels for each dilution tested. Results of four such tests with *R. rickettsi*, performed at different times, yielded nearly identical plaque values in both systems. Plaque values with *R. typhi* also were nearly equal in both systems (Table 2).

Comparative tests with the other two agents

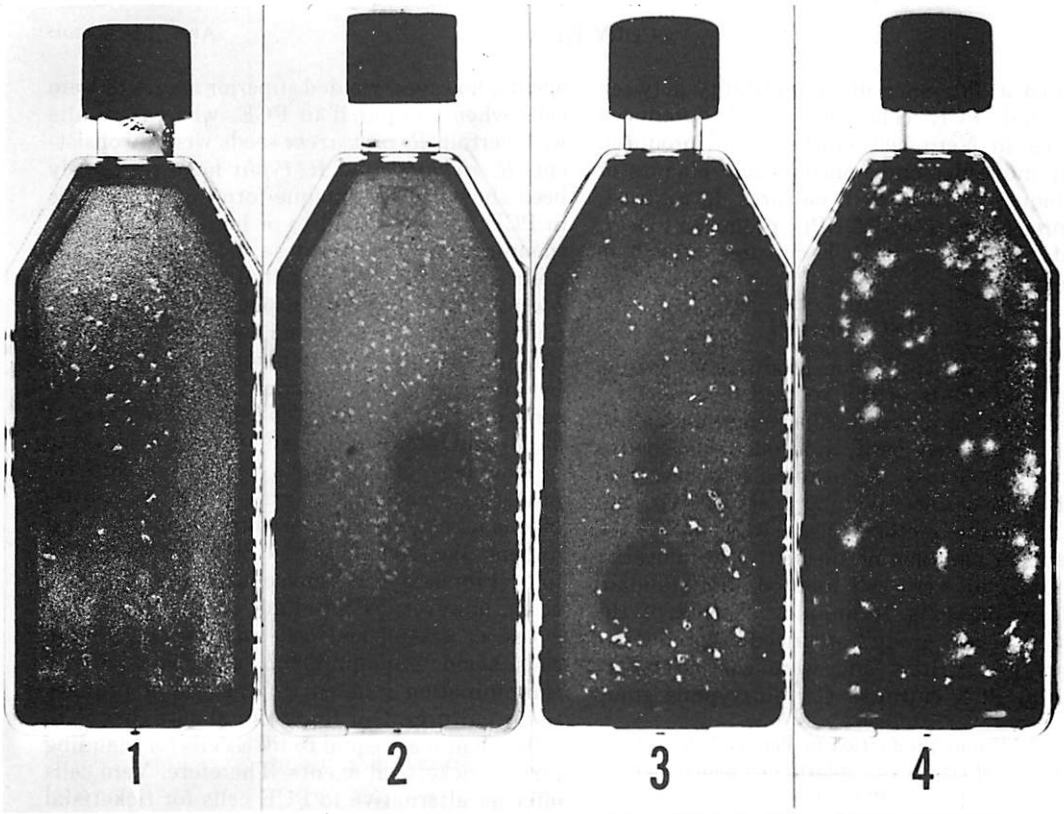

FIG. 1. *Plaque production in Vero cells by certain rickettsiae: (1) Rickettsia typhi stained after 20 days of incubation at 27 C; (2) R. montana after 8 days at 37 C; (3) R. conori after 8 days at 37 C; (4) R. canada after 12 days at 35 C.*

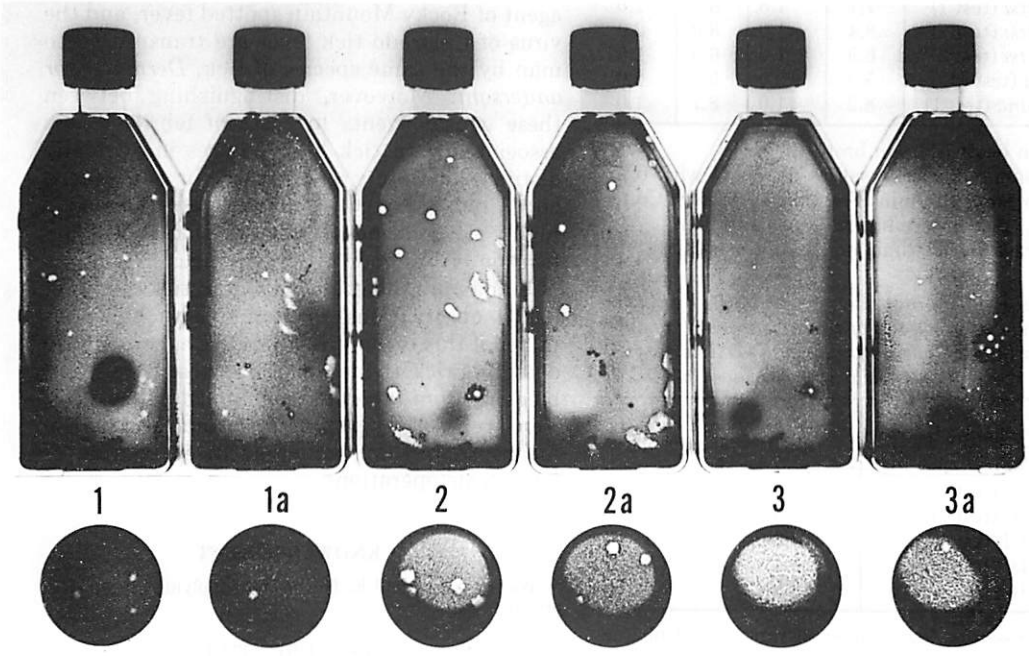

FIG. 2. *Rickettsia rickettsi plaques in Vero cells. Effects of vessels (above, flasks; below, same in trays); different overlay media; and addition of 10 mM HEPES buffer. (1) Medium 199; (1a) same, with HEPES buffer; (2) Leibovitz medium; (2a) same, with HEPES; (3) Eagle minimum essential medium; (3a) same, with HEPES. All incubated for 7 days at 32 C.*

revealed a difference in susceptibility between Vero and PCE cells. *R. canada* produced plaques in Vero cell cultures but produced either indistinct and noncountable plaques or no plaques at all in PCE cultures. In contrast, *R. prowazeki* (15GP/2EP) produced clear, countable plaques in PCE cultures but not in Vero cells.

DISCUSSION

We have shown plaque production with six rickettsial agents of the spotted fever and typhus groups in an established mammalian cell line (Vero) widely used, especially in arbovirus studies. Previously, *R. rickettsi* had been plaqued in this line (Weinberg et al. [4]), but details of the methods used were not given. Except for omission of antibiotics from overlay medium, our Vero cell methods are standard virologic plaquing techniques. Agents of the spotted fever group produced reproducible plaque assay titers and were comparable to those in PCE cultures. Certain typhus group agents, however, yielded superior assays in Vero cells when compared to PCE, whereas results with certain *R. prowazeki* seeds were inconsistent. *R. rickettsi* and *R. typhi* have previously been shown to give plaque-forming unit values in PCE cultures equal to or higher than corresponding mean lethal dose or mean infective dose values in embryonated eggs (4, 6). *C. burneti* failed to produce plaques in Vero cells under the conditions tested, whereas Wike et al. (6) induced this agent to plaque in PCE culture by modifications of overlay medium and extended incubation. BHI, as used by Weinberg et al. (4) and later determined by Wike et al. (5) to be the best of numerous diluents tested for preparing rickettsial inocula for PCE culture plaque assay, was compared to a standard viral diluent. We found 0.15 M PBS + BPA superior to BHI for assays in Vero cells. As used by Wike et al., however, PBS did not contain BPA.

Use of established cell lines, such as Vero cells, avoids some problems of preparation and contamination inherent in the use of primary tissue cultures. Our findings indicate that Vero cells are at least equal to PCE cells for plaquing certain rickettsial agents. Therefore, Vero cells offer an alternative to PCE cells for rickettsial plaque assays. In addition, Vero cells may provide a practical and economical means to test for more than one infectious agent in a single sample. Both *R. rickettsi*, the causal agent of Rocky Mountain spotted fever, and the virus of Colorado tick fever are transmitted to man by the same species of tick, *Dermacentor andersoni*. Moreover, distinguishing between these causal agents in cases of febrile illness associated with tick bite requires individually distinct laboratory procedures. In our experience, the Vero cell plaquing technique is a highly efficient method for isolation and identification of Colorado tick fever virus in samples of human or rodent bloods and in ticks (unpublished observations). It remains to be determined if this technique will prove as sensitive in detecting rickettsiae from clinical or field specimens as from laboratory-produced seeds. If so, Vero cells offer a practical and economical means to test for either agent in routine or large-scale operations.

TABLE 1. *Plaque production in Vero cells by certain rickettsiae: effects of two diluents on plaque number and size*

Seed	BHI[a]		PBS + BPA[b]	
	PFU[c]	Size[d]	PFU[c]	Size[d]
R. rickettsi (test 1)	7.7	1.5	8.0	2.0
R. rickettsi (test 2)	8.4	1.0	8.9	2.0
R. rickettsi (test 3)	6.9	1.0	6.9	1.5
R. conori (test 1)	5.3	2.0	5.5	2.0
R. montana (test 1)	8.2	<1.0	8.7	1.0

[a] Brain heart infusion broth (Difco).
[b] Phosphate-buffered saline (0.15 M) with 0.75% bovine plasma albumin (Fraction V, Armour Co.).
[c] $\log_{10}$ plaque-forming units per milliliter.
[d] Average plaque diameter in millimeters.

TABLE 2. *Plaque production in Vero cells by certain rickettsiae: comparison of titers between Vero cells and primary chick embryo (PCE) cell cultures receiving identical yolk sac inocula*

Seed	Vero (PFU)[a]	PCE (PFU)[a]
R. rickettsi (test 1)	7.9	7.9
R. rickettsi (test 2)	7.9	7.7
R. rickettsi (test 3)	7.3	7.8
R. rickettsi (test 4)	8.4	8.2
R. typhi (test 1)	8.5	8.4

[a] Expressed as $\log_{10}$ values of numbers of plaques per gram of yolk sac tissue. Each assay was performed with two to four cell culture vessels for each dilution tested.

ACKNOWLEDGMENT

We thank Lyndahl E. Hughes for supplying certain rickettsial seeds.

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ISOLATION OF SPOTTED FEVER GROUP RICKETTSIA FROM PATIENTS WITH FEBRILE EXANTHEMATOUS ILLNESS BY USE OF VERO CELLS

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Rickettsial infections of the spotted fever group (SFG) were newly found in 1984 in areas along the coast from Anan city to the Muroto promontory, *i.e.*, the southeast coast of Shikoku, one of Japan's four principal islands (1). Sera from patients with a characteristic febrile exanthematous illness reacted with *Rickettsia rickettsii* and *R. akari* in the complement fixation test (1), with *R. sibirica* and Thai-tick typhus rickettsia in the immunoperoxidase test (2), and with *R. montana* in the immunofluorescence (IF) test (3).

Isolation of the causative agent was carried out in 1985 by a cell culture technique from patients who had fever, rash, and an eschar (4). The patients' blood was collected prior to tetracycline treatment at the Muroto Hospital, immediately heparinized, and quickly frozen in an alcohol-dry ice bath. The blood specimens were transported to the laboratory and stored at $-85°C$. To a tube containing 1 ml of a frozen blood specimen, an equal volume of chilled heparin solution (20 u/ml) of phosphate buffered saline devoid of Ca^{2+} and Mg^{2+} was added, and the blood was thawed and subjected to centrifugation at 3,500 rpm for 30 min at 2°C. The sediment was resuspended in 0.2 ml of Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) without antibiotics. Then 0.1 ml of the suspension was spread over a two-day-old confluent monolayer of Vero cells grown in 25-cm^2 tissue culture flasks. The flasks were kept for 1 hr at 34°C. After the addition of 5 ml of MEM containing 10% FBS, the loosely capped flasks were placed in a CO_2 incubator at 34°C. The following day the medium was replaced by MEM containing 2% FBS, and the incubation was continued with medium changes every 3 or 4 days. After 10 days of incubation, many granules or vesicles that looked like small rounded cells were observed over the monolayer under a microscope with a magnification of 40x. No destructive changes had occurred in the cell monolayer. The cells were detached with trypsin and sodium ethylenediaminetetraacetate (EDTA) and collected by low-speed centrifugation. Half of the sedimented cells were plated with uninfected Vero

cells and the other half were plated alone. In both cases the cells grew well and formed monolayers. A week later, the cells were detached with trypsin and EDTA and centrifuged down for further cultivation. Smears of cells from a part of the sediment were made on slides, heat-fixed, and stained by the Giménez method. It was found that all of the cells contained short rod-shaped organisms staining brilliant red. The smears were also fixed in acetone for 30 min at 4°C then subjected to an indirect IF test with the convalescent-phase serum and fluorescein isothiocyanate-labeled anti-human IgG antibody. Brightly stained particles in the cells were observed under a fluorescence microscope. The cells carrying organisms were passaged every 3 days. Vero cells harboring the organisms are shown in Fig. 1. In Fig. 2, rickettsiae in cells are visualized by the bright yellowish green fluorescence of the fluorescein conjugate. The locali-

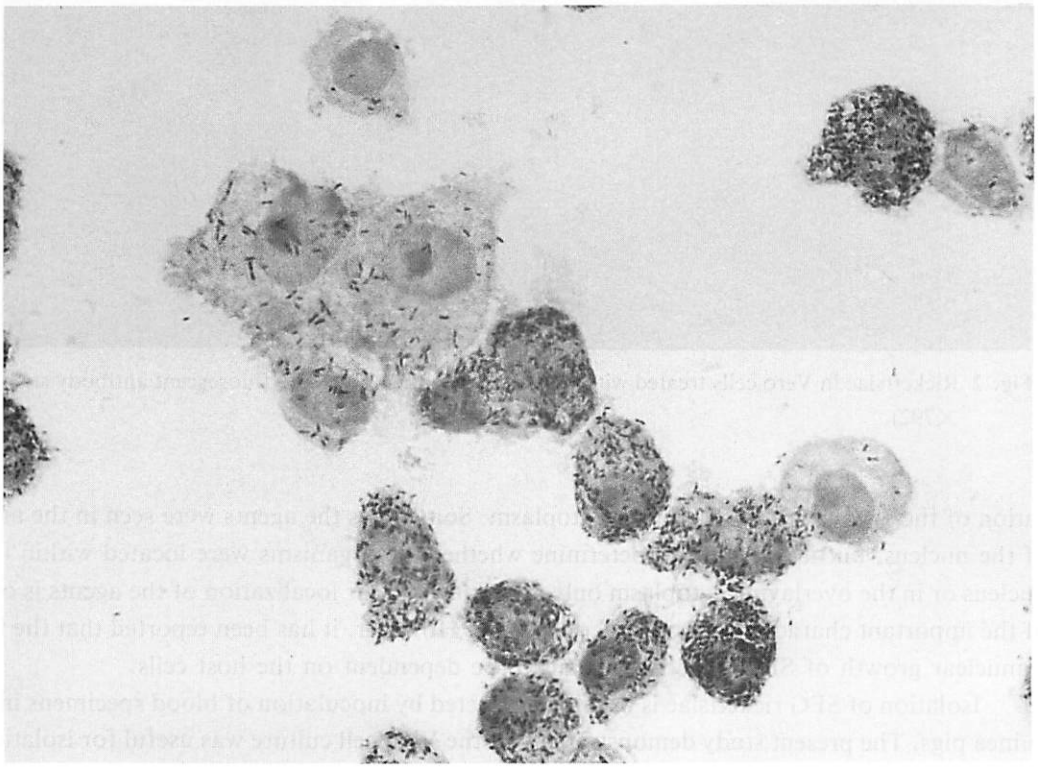

Fig. 1 Isolated rickettsiae grown in Vero cells (Giménez stain, ×792).

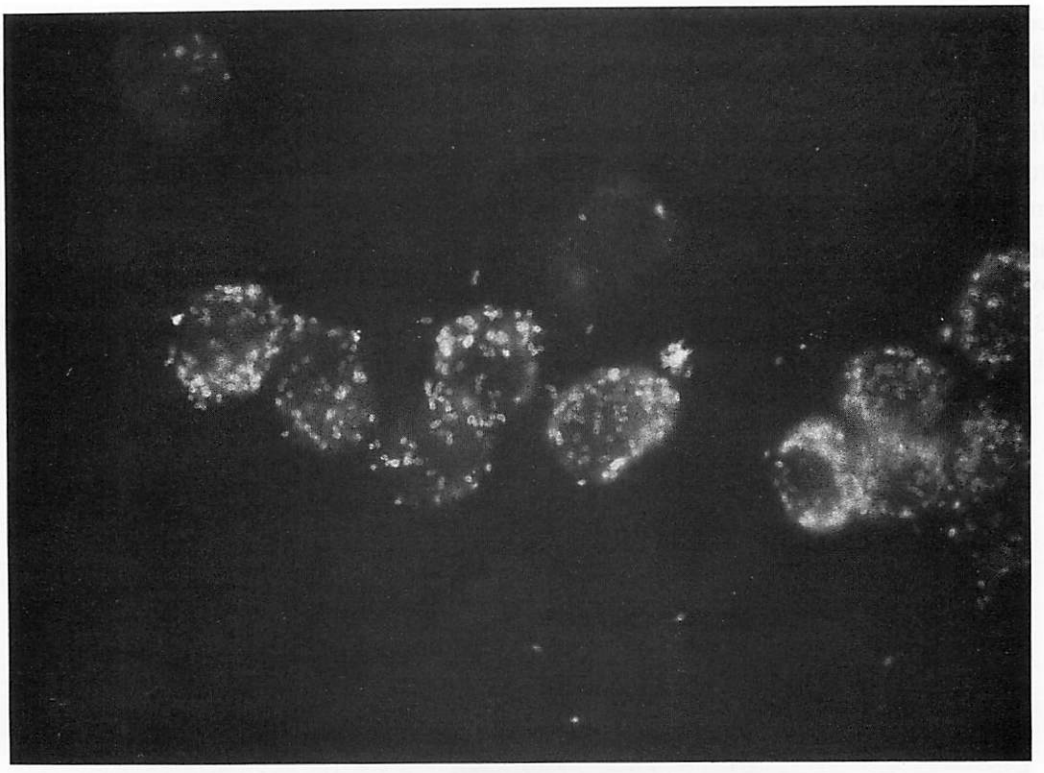

Fig. 2 Rickettsiae in Vero cells treated with homologous patient's serum (fluorescent antibody stain, ×792).

zation of the agents was mainly in the cytoplasm. Sometimes the agents were seen in the area of the nucleus, but it was hard to determine whether the organisms were located within the nucleus or in the overlaying cytoplasm only. The intranuclear localization of the agents is one of the important characteristics of SFG rickettsiae. However, it has been reported that the intranuclear growth of SFG rickettsiae seems to be dependent on the host cells.

Isolation of SFG rickettsiae is usually conducted by inoculation of blood specimens into guinea pigs. The present study demonstrated that the Vero cell culture was useful for isolation of SFG rickettsiae, while BHK and other cell lines tested were not suitable.

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6.

VERO Cells for Bacterial Toxin Study

Introduction

Toxins produced by bacteria have been playing pioneering roles in the study of serologic immunology since the discovery of the diphtheria toxin and antitoxin. Experimental animals are known to be specifically affected by a minute amount of a bacterial toxin. Thus, in the past, these animals have been used in the study and evaluation of the effectiveness of the toxin. However, now, with the advancement of cell biology, tissue cultures are being used instead. In 1974, the diphtheria toxin was found to be specifically cytotoxic to Vero cells. The effectiveness of this toxin was dose dependent and was neutralized by antitoxin. After various types of cultured cells were examined, Vero cells were found to be the most susceptible to the toxin. As a result, a quantification system was established for the measurement of diphtheria toxin and antitoxin using Vero cells in a microplate culture system similar to that utilized in virology studies (p. 161). This method is currently used as the standard procedure for sero-epidemiologic surveillance of diphtheria antitoxin in Japan. In addition, Vero cells are used as the reference indicator during titration assays in potency tests for toxoid tablets.

More recently, Vero cells have been used for the basic study to elucidate the mechanisms of cell damage by toxins. Enterotoxin, produced by *Clostridium perfringens*, greatly damages the cytoplasmic membrane, its mechanism of action is now being studied using Vero cells.

It has long been known that *Shigella dysenteriae* produces a toxin that is known by various names. Recently, it was determined that measurement of the lethal dose of Shiga toxin against Vero cells is the most sensitive technique available to measure the biological effect of Shiga toxin. The LD_{50}, that is the dose needed to kill 50% of the cells, for Vero cells is 1 pg. When 10 pg of the toxin were used, all of the cells were killed. Shiga toxin also has a similar toxic effect on a specified strain of HeLa cells; however, most HeLa cells are unaffected by this Shiga toxin.

In 1978, a toxin produced by a type of enterobacteria (*E. coli* 0157:H7) was found to be fatally toxic to Vero cells (p. 175). This finding did not attract much attention at the time. However, in 1982, several cases of food poisoning in the United States of America were found to be caused by enterobacteria. The isolated bacteria, *E. coli* 0157:H7, produced a *Verotoxin* which kills Vero cells efficiently and thus attracted a great deal of attention. Since then, a number of cases of food poisoning caused by this bacteria have been reported throughout the world. The major symptoms resemble those of hemorrhagic colitis. From an immunological viewpoint, the *Verotoxin* exhibits some of the same characteristics as the toxin released by *Shigella dysenteriae*, and both are found to contain an identical protein. Thus, the new toxin became known as the Shiga-like toxin. When the LD_{50} of this toxin was established, it was found to be the same as that of Shiga toxin. Recently, using tests involving Vero cells, it was discovered that there are definitely two types of toxins produced by *E. coli.* 0157:H7. One of these is the toxin described above, the Shiga-like toxin I. The other completely lacks characteristics of Shiga toxin from an immunological viewpoint, although it is occasionally referred to as the Shiga-like toxin II. Both of these toxins are found to inhibit protein synthesis in the eukaryotic cell. Based on these findings, a new direction has been taken in the study of bacterial toxins using tissue cultures.

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Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells

I. Studies on factors affecting the toxin and antitoxin titration*

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A. Ito,‡ *R. Murata*‡ *and R. Kono*†

A micro cell culture system was applied to diphtheria toxin and antitoxin titrations using a colour change of phenol red as an indicator of the toxin activity on the cells. One of the characteristics of the present method is to seed cells simultaneously with either toxin or toxin–antitoxin mixture. The end point of the toxin action was shown to be inversely proportional to the cell concentration. The authors adopted a seed of 1×10^4 cells per well of the microplate in the standardized procedure. The calf sera were screened for their diphtheria antitoxin activity and those which showed no neutralization were used in the tests in cell cultures.

The results were highly reproducible in the determination of the minimum cytopathic dose (MCD) by the micro cell culture method and the MCD values obtained were almost identical to the minimal reactive dose (MRD) as determined by the rabbit skin test.

The neutralization of toxin by antitoxin followed the law of multiple proportions at the concentrations tested as well as at very low levels. The minimum level of antitoxin detectable by this system was 0·005 iu/ml when determined with 4 MCD of the working crude toxin but could be lowered to 0·00125 iu/ml by challenging with a newly prepared test toxin. A lower antitoxin level was also shown to be detectable by increasing the volume of antiserum.

Since a complete correlation was established between two antitoxin titres of sera examined by crude and highly purified toxin preparations simultaneously, it was concluded that the working crude toxins may be used for the antitoxin titration in the present micro cell culture method.

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INTRODUCTION

The cytotoxicity of diphtheria toxin was established in cultured chick embryo cells by Levaditi & Mutermilch (1913) and Okabe (1933) suggested that such cultures might be of value in titrating diphtheria antitoxin. In 1957 Placido-Sousa & Evans developed a method of quantitative titrations of diphtheria toxin and antitoxin by primary culture of monkey kidney cells and reported that the colour change of phenol red in the culture medium could be a useful indicator of the cytotoxicity. Thereafter several authors reported the availability of various cell cultures and modifications of the procedure to obtain a practical application (Topciu, Babuşceac-Plavoşin & Nica, 1966; Jandásek & Svobodova, 1967; Kříž, Vysoká-Burianová, Žáček, Teplý & Burian, 1967; Izbický, 1968, 1969).

Microtitre systems as well as the microplate cultivation of cells have developed during the last decade in the field of virology for viral titration and neutralization tests (Sever, 1962; Catalano, Fuccillo & Sever, 1969). The new methods allow an enormous number of samples to be tested simultaneously with minimum effort and materials. Quevillon & Chagnon (1973) reported recently that the microtissue culture technique could be applied to the titration of diphtheria toxin and antitoxin.

Independently we have developed a different micromethod for the titration of diphtheria toxin and antitoxin using microplate cultures of VERO cells. The reason why we employed VERO cells was that they were proved to be as sensitive as human cells to diphtheria toxin by Greaves, Potter & McEntegart (1971) and were easily handled in the laboratory. Our method has two distinct advantages: it is time-saving because VERO cells are seeded in wells of the microplate simultaneously with the toxin or toxin–antitoxin mixtures, transfer plates are employed for neutralization and the pH colour change is adopted for the reading of the end points instead of the cumbersome microscopic observations. Furthermore, this method has been shown to be easier than and as accurate as the rabbit skin test. This first report will be concerned with the investigation of factors influencing the test and the establishment of a standard method.

MATERIALS AND METHODS

Diphtheria toxins

Three lots of crude toxin preparations were employed for the test toxin. Two of them, No. 59 and No. M-46, were matured by long storage whereas the third No. 223 was prepared recently. Lf titres of these toxins were 38, 110 and 110 per ml, respectively. In addition to these a highly purified toxin preparation, having a purity of 3300 Lf per mg protein nitrogen, was kindly supplied by Dr I. Kato, of the Institute of Medical Science, the University of Tokyo, and was included in the test.

Standard diphtheria antitoxin

The National Standard Antitoxin was used as the standard. This is a hyperimmunized horse antitoxin and is distributed as a solution, containing 10 International Units (iu) per ml in glycerine–saline (2 : 1).

Human immune sera

Sera from 12-year old children were obtained just before and six to eight weeks after the third booster injection with diphtheria–tetanus toxoids (D–T).

Guinea-pig immune sera

Guinea-pigs were immunized with the National Standard Diphtheria Toxoid or D–T–P vaccines subcutaneously and bled four weeks after the injection.

Rabbit skin test

The method as described by Jensen (1933) was used.

Micro systems

Two kinds of plastic microtissue culture plates (microplates) having 96 flat-bottomed circular wells were used; these were the Micro test II plates (Falcon Plastics) and the Microtiter Plates (Cooke Engineering Co., Ltd). Transfer plates (Cooke Engineering Co., Ltd) and pressure sensitive film (Falcon Plastics) were used in the dilution and neutralization procedures, and in sealing the plates, respectively. Twenty-five microlitre droppers were used for dispensing the materials and 25 microlitre diluters were used for serum dilution in transfer plates.

Cell cultures

VERO cells passaged in Eagle's minimum essential medium (MEM) enriched with 10% calf serum were digested with 0·25% trypsin in phosphate buffer solution (PBS) containing 0·01% EDTA. The dispersed cells were centrifuged and resuspended to 2×10^5/ml of viable cells. A 0·1% erythrosin B solution in phosphate buffered saline (PBS) was used in viable cell counts.

Medium for the pH colour change method

A modified Eagle's MEM in which the concentrations of both phenol red and glucose were increased to 0·003 and 0·4%, respectively, was used as the growth medium after the addition of sodium bicarbonate and calf serum to give a final concentration of 0·15 and 2%, respectively. The medium was used also as the diluent for the toxin, the standard antitoxin and the sera to be tested. The calf serum in the cell cultures was tested for the presence of antitoxic activity and only those sera free from such activity were used.

Determination of minimum cytopathic dose (MCD) of the toxin

Twenty-five microlitres of each serial dilution of toxin, diluted by twofold or by 30% increment steps, were transferred to each of two wells of a microplate. To each well was added 125 μl of medium and 50 μl of VERO cell suspension (2×10^5 cells/ml). The plates were sealed with pressure-sensitive films and were incubated at 37 °C for 4–5 days. As the colour of the medium in the wells in which the cellular metabolism was stopped by the toxic action remained red, the highest dilution of the toxin in which there was no colour change was taken as 1 MCD of toxin.

Assay of the antitoxin titre

In the experiments to determine the standard conditions for the antitoxin assays the standard antitoxin or sera to be assayed were diluted in serial twofold steps or 30% increments in tubes. These dilutions (25 μl) were then transferred to wells of a microplate and an equal volume of challenge toxin was added. The plate was incubated for 60 min at 37 °C before adding 100 μl of culture medium and 50 μl of a VERO cell suspension (usually 2×10^5 cells/ml except in special experiments indicated in the text).

For the routine procedure of the antitoxin titration of test specimens, a standard method using a transfer plate was developed: 25 μl of diluent is dropped in each well except for those in the two upper horizontal rows (see Plate 1 for an example of a typical layout). Twenty-five μl of a serum specimen is placed in each well of the first three rows and twofold dilutions of each serum are made with a microdiluter from the third to the eighth row. Twenty-five μl of the toxin (containing not less than 4 MCD) is distributed to each of the wells except those in row 1, which have culture medium instead of the toxin and which serve as the serum control for the growth of the cells. The transfer plate is then shaken for a few minutes and incubated for 60 min at 37 °C.

A hundred μl of culture medium are distributed into each well of the microplates and then the transfer plate is placed on it to allow the serum toxin mixtures to pass into the microplates. Finally, 50 μl of VERO cell suspension (2×10^5 cells/ml) are distributed into each well of the microplate.

Each run of the test involves the following controls: (1) dilutions of the standard antitoxin, (2) retitration of the test dose of the toxin and (3) twofold dilutions of the cell suspension. All the microplates are sealed with pressure-sensitive films and incubated for a few days at 37 °C.

The result is read at the time when the culture medium in the wells containing the cell control has changed colour from red to yellow, and at this time the end point of titration is taken as the highest dilution of serum in the well in which the colour of the culture medium is also yellow. The antitoxin titres are expressed in iu by comparison with the result of the standard antitoxin.

A flow sheet of the standardized routine procedure of diphtheria antitoxin titration is shown in Fig. 1.

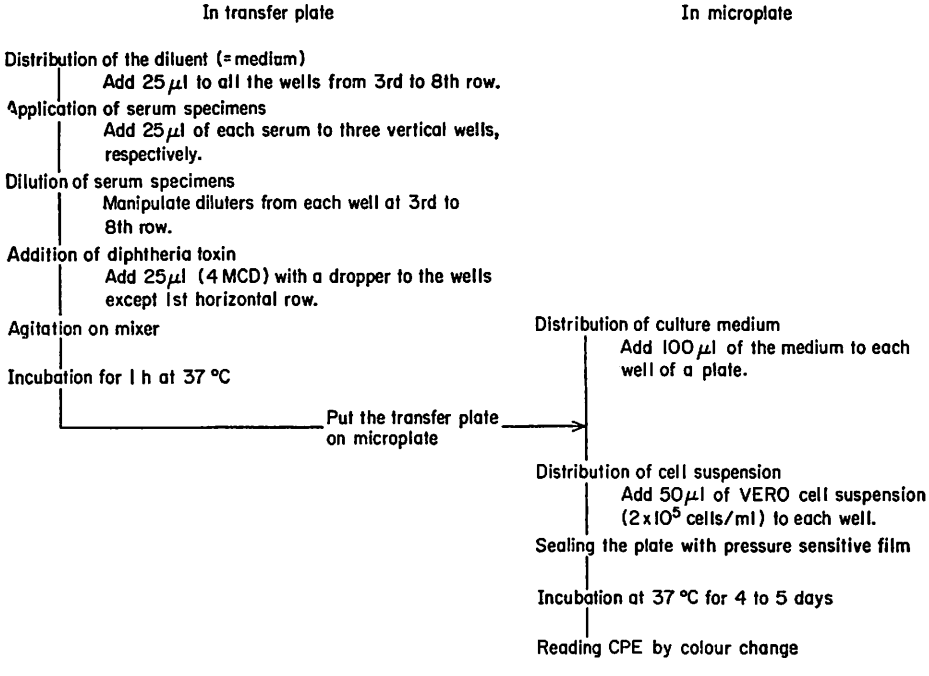

Fig. 1. Flow sheet of the micro cell culture colour change method for diphtheria antitoxin titration.

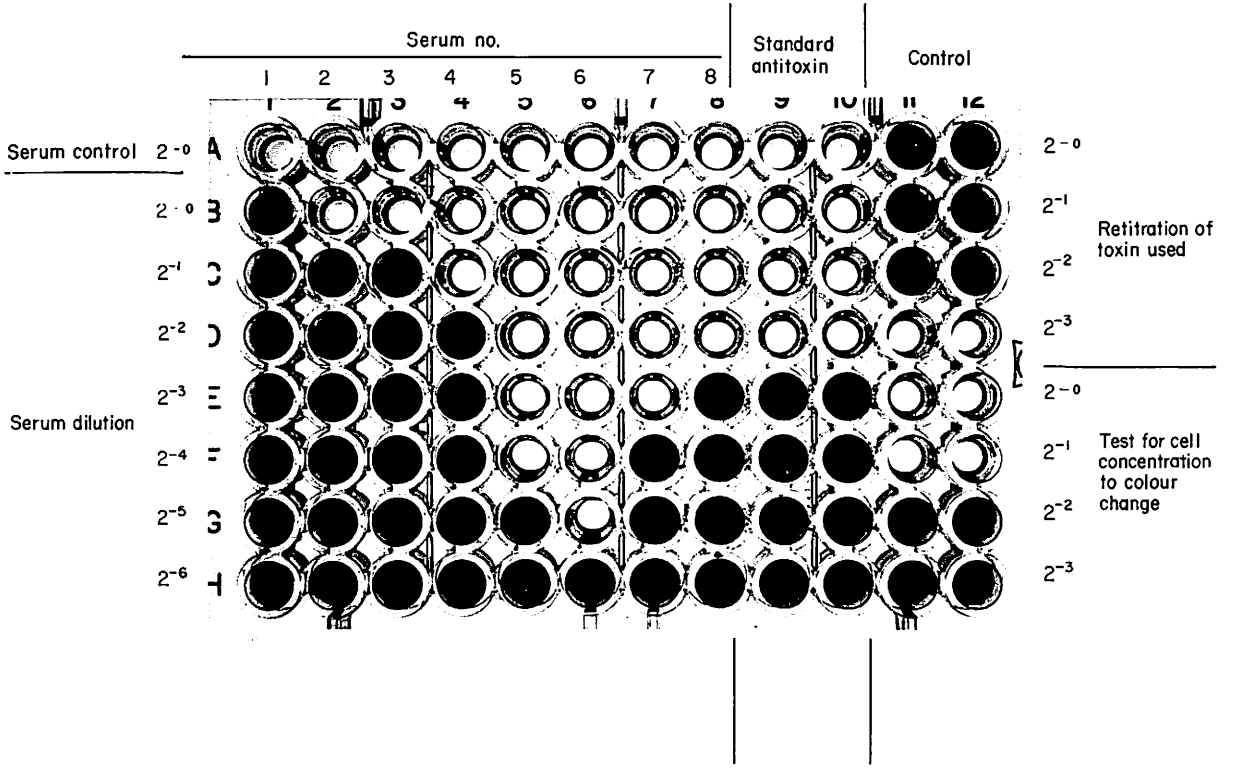

Plate 1. Typical layout for antitoxin titration.

RESULTS

Presence of diphtheria antitoxic activity in calf sera used for cell culture

Twenty-one lots of calf serum were tested for their antitoxic activity to diphtheria toxin. A hundred μl of 20% calf serum solution were dropped in each well of the microplate. Twofold serial dilutions were made of diphtheria toxin No. 59 which had already been diluted 1 : 100. Fifty μl each of these twofold dilutions (ranging from 1 : 400 to 1 : 51 200) were added to the wells in horizontal rows. Finally, 50 μl of a VERO cell suspension (2×10^5 cells/ml) were added to each well and the microplate was sealed with pressure-sensitive film and incubated at 37 °C .

Toxin No. 59 showed CPE up to a dilution of 1 : 25 600 when calf sera, which had been shown to be free from antitoxic activity by the rabbit skin test, were used in the cell cultures. However, the addition of some calf sera, at a final concentration of 10%, lowered the end point to 1 : 400. When compared with the results obtained with a standard antitoxin, the antitoxin activities of several lots of calf sera were as follows: 12 of 21 lots were shown to be free of antitoxic activity, some of the 21 lots neutralized more than 1 MCD of toxin and the serum with the greatest antitoxic activity had a titre of 0·08 iu/ml. Only the 12 lots with no antitoxic activity were used in the tests.

Effect of cell concentration on toxic titration

Two toxins were diluted by 30% increments in each dilution. Twenty-five μl of each dilution of the toxins were distributed in the wells in the vertical rows of the microplate. On the same microplate cell suspensions having different cell concentrations from 2×10^4 to 5×10^3 cells/well were added to each well in the horizontal rows and

TABLE 1. Effect of VERO cell concentration on MCD. Activities of two toxin lots with various cell concentrations were shown as MCD $\times 10^4$ per 25 μl

Toxin	Cells seeded per well ($\times 10^4$)					
	2·0	1·5	1·25	1·0	0·75	0·5
No. 59	0·94	1·33	2·00	2·00	5·32	5·32
No. M-46	1·31	1·92	2·70	2·70	3·76	5·32

incubated at 37 °C for several days. It was shown (see Table 1) that the end points of toxin activity were inversely proportional to the cell concentration.

Effect of concentration of calf serum in the cell culture medium

Both toxins as used in the previous experiment were included and a constant concentration of 1×10^4 viable cells per well was used. Calf serum which had been shown to be free from antitoxic activity was added to give final concentrations of 2, 5 and 10%. The toxins No. 59 and No. M-46 showed an activity of $2{\cdot}0 \times 10^4$ MCD and $2{\cdot}7 \times 10^4$ MCD per 25 μl, respectively, in the medium containing 2% calf serum but the toxicities were enhanced to $2{\cdot}6 \times 10^4$ and $3{\cdot}8 \times 10^4$ MCD per 25 μl when the calf serum concentration was increased to 5% or more. This result was repeatedly confirmed, although the reason for it is unknown.

Diphtheria toxin titres determined by minimum cytopathic dose (MCD) in cell cultures and the minimal reactive dose (MRD) in the skin of rabbits

Three unpurified lots and one highly purified lot of diphtheria toxin were diluted by 30% increments and the MCD and MRD values determined. In the MCD titration volumes of 25 μl were used of each toxin dilution in each well and the highest dilution of the toxin in which the colour of the medium remained red after incubation was taken as 1 MCD. Three replicate experiments gave identical MCD titres for each toxin preparation (see Table 2). The geometric mean titres for the toxins No. 223 and M-46 gave ratios of MCD/MRD as low as 0·74–0·47, respectively, whereas the ratio for toxin No. 59 and the purified toxin was unity.

Comparison of the neutralizing activity of the standard antitoxin by box titration using three toxin preparations

The standard antitoxin which had been diluted to contain 0·04 iu/ml was diluted further by 30% increments and 25 μl of each dilution was distributed to wells in the vertical rows. The same volumes of 30% increment dilutions of three lots of toxins were distributed into wells containing antitoxin in the horizontal rows. Thus the box titration was completed two-dimensionally.

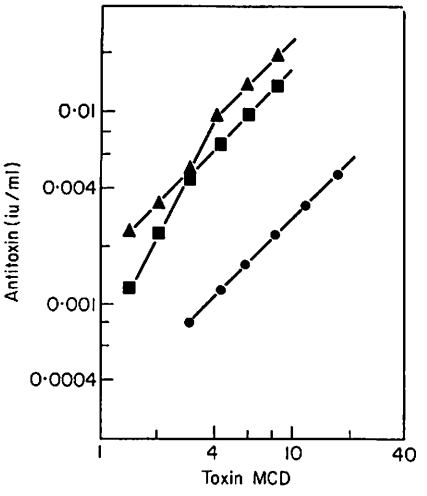

Fig. 2. Neutralization curves of the standard antitoxin tested with three toxin preparations. (▲) Toxin No. M-46; (■) toxin No. 59; (●) toxin No. 223.

Fig. 2 shows the amounts of the standard antitoxin in iu required to neutralize various challenge doses of the test toxins. Three parallel straight lines were obtained and the distance between then appears to represent the difference in the toxoid content of each toxin preparation; that is, No. M-46 and No. 59 may contain more toxiod than No. 223 which was prepared at a later date. This concept is supported also by the ratios between the MCD and Lf titre obtained in three different toxins as shown in Table 2. Therefore, it may be concluded that freshly prepared toxin is more effective in the titration of lower antitoxin titre than older toxin preparations.

It was observed that the lines deviated from a straight line at the lower limits of the toxin. This deviation may not necessarily mean a change in reactivity at the lower

TABLE 2. Activity of four diphtheria toxins determined by the micro cell culture method and rabbit skin test

Toxin	Lf/ml	MRD/ml ($\times 10^5$)	MCD/ml ($\times 10^5$) Test 1	Test 2	Test 3	Geometric mean	MCD/Lf ($\times 10^5$)	MCD/MRD
No. 59	38	5·2	5·3	5·3	6·0	5·53	0·146	1·06
No. M-46	110	25·0	10·6	10·6	14·3	11·71	0·106	0·47
No. 223	110	230	170	170	170	170	1·545	0·74
Purified toxin	2500	4600	5100	4800	—	4948	1·979	1·08

TABLE 3. Theoretically calculated residual MCDs in each well of three types of box titration using different serial dilutions. (a) 30% increment dilution, (b) twofold dilution, (c) dilution by 0·25 interval

(a)

Toxin MCD	MCD equivalent antitoxin 12·14	8·50	5·95	4·16	2·91	2·04	1·43	1·00	0·70	0·49	0·34	0·24
12·14	0	3·6										
8·50		0	2·6									
5·95			0	1·8								
4·16				0	1·3	2·1						
2·91					0	0·9	1·5					
2·04						0	0·6	1·0	1·3	1·6		
1·43							0	0·4	0·7	0·9	1·1	1·2
1·00								0	0·3	0·5	0·7	0·8

(b)

Toxin MCD	MCD equivalent antitoxin 128	64	32	16	8	4	2	1	0·50	0·25	0·13	0·06
128	0	64										
64		0	32									
32			0	16								
16				0	8							
8					0	4						
4						0	2					
2							0	1	1·5	1·75	1·88	1·93
1								0	0·5	0·75	0·88	0·93

(c)

Toxin MCD	MCD equivalent antitoxin 2·75	2·50	2·25	2·00	1·75	1·50	1·25	1·00	0·75	0·50	0·25	0·00
2·75	0	0·25	0·50	0·75	1·00							
2·50		0	0·25	0·50	0·75	1·00						
2·25			0	0·25	0·50	0·75	1·00					
2·00				0	0·25	0·50	0·75	1·00				
1·75					0	0·25	0·50	0·75	1·00			
1·50						0	0·25	0·50	0·75	1·00		
1·25							0	0·25	0·50	0·75	1·00	
1·00								0	0·25	0·50	0·75	1·00

concentrations of the toxin, but may be explained in the following way. Table 3 shows the theoretically calculated values of residual toxin after neutralization with an antitoxin. For the convenience of calculation, that amount of antitoxin (in units) which neutralizes 1 MCD of toxin are referred to as 1 MCD equivalent of antitoxin. The residual toxin in each well was also calculated and expressed on a MCD basis by diminishing the MCD equivalent antitoxin value from the MCD of the toxin added to each well. The deviation from the straight line occurs when a dilution interval of the antitoxin is less than 1 MCD equivalent, as shown in Table 3(a) and (b). The inflexion point appeared when the toxin dose was less than 4 MCD in the 30% increment dilution method or 2 MCD with the twofold dilution method, but the deviation did not appear when an interval of 0·25 was chosen. The explanation above was further confirmed by the following model experiment in which the standard antitoxin was diluted between the concentrations of 1·90 and $0{\cdot}22 \times 10^{-3}$ iu/ml and challenged two-dimensionally with toxin of less than 3 MCD. In Table 4(a) the actual colour changes are

TABLE 4. Toxin–antitoxin reactions at low levels. (a) Actual colour change pattern in the experiment; (b) calculated MCD values remaining in each well in (a), when assumed that $1{\cdot}25 \times 10^{-3}$ iu/ml was the 1 MCD equivalent of the antitoxin

(a)

Toxin MCD	Antitoxin iu/ml $\times 10^{-3}$									
	1·90	1·25	0·95	0·63	0·55	0·48	0·39	0·32	0·26	0·22
3·00	R*	R	R	R	R	R	R	R	R	R
2·50	R	R	R	R	R	R	R	R	R	R
2·00	Y	R	R	R	R	R	R	R	R	R
1·75	Y	Y	YR	R	R	R	R	R	R	R
1·50	Y	Y	Y	YR	YR	YR	R	R	R	R
1·25	Y	Y	Y	Y	Y	Y	YR	R	R	R
1·00	Y	Y	Y	Y	Y	Y	Y	YR	R	R

(b)

Toxin MCD	MCD equivalent antitoxin									
	1·50	1·00	0·75	0·50	0·44	0·38	0·31	0·25	0·21	0·18
3·00										
2·50	1·00	1·50								
2·00	0·50	1·00	1·25							
1·75		0·75	1·00	1·25						
1·50			0·75	1·00	1·06	1·12	1·19			
1·25				0·75	0·81	0·87	0·94	1·00	1·04	1·07
1·00							0·69	0·75	0·79	0·82

* Colour in each well: Y = yellow, YR = orange, R = red.

shown of the pattern obtained on a microplate and Table 4(b) shows the MCD values of the toxin remaining in each well; each figure was theoretically calculated in an identical manner to that used in Table 3. As one can see (Table 4) the colour change pattern in Table 4(a) represented an exact copy of that expected [shown in Table 4(b)] except for the lowest row in the Table. Thus it seems that the toxin–antitoxin reaction takes place proportionally in this system and, theoretically, it may be possible to titrate much

lower levels of antitoxin if the amount of the test toxin is reduced to a level lower than 4 MCD used routinely and also if the serum dilutions are made in an arithmetic progression as in Table 3(c) in which the differences chosen are 0·25.

Antitoxin titres determined at different challenge doses of the test toxin

As shown in Fig. 2, the reaction between diphtheria toxin and antitoxin follows the law of multiple proportion and the antitoxin titre obtained was identical whatever level of toxin was used provided that it fell within the range of the straight line portion of the neutralization curve. Higher challenge doses were also tested.

TABLE 5. Antitoxin titres of 10 sera determined at two different challenge doses of the toxin. (a) Challenge with 4 MCD, (b) challenge with 256 MCD

(a)

Serum dilution	Serum number 1	2	3	4	5	6	7	8	9	10	Standard antitoxin	St. AT. (iu/ml)
1 : 64	Y*	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y Y	0·010
1 : 128	R	R	R	Y	Y	Y	Y	Y	Y	Y	Y Y	0·005
1 : 256	R	R	R	R	Y	Y	Y	Y	Y	Y	R R	0·0025
1 : 512	R	R	R	R	Y	Y	Y	Y	Y	Y	R R	0·00125
1 : 1024	R	R	R	R	R	R	R	YR	YR	YR	R R	0·00063
1 : 2048	R	R	R	R	R	R	R	R	R	R	R R	0·00031
1 : 4096	R	R	R	R	R	R	R	R	R	R	R R	0·00016
1 : 8192	R	R	R	R	R	R	R	R	R	R	R R	0·00008
Antoxin titre of each serum	0·32	0·32	0·32	0·64	2·56	2·56	2·56	5·12	5·12	5·12	0·64	

(b)

Serum dilution	Serum number 1	2	3	4	5	6	7	8	9	10	Standard antitoxin	St. AT. (iu/ml)
1 : 1	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y Y	0·64
1 : 2	R	R	R	Y	Y	Y	Y	Y	Y	Y	Y Y	0·32
1 : 4	R	R	R	R	Y	Y	Y	Y	Y	Y	R R	0·16
1 : 8	R	R	R	R	Y	Y	Y	Y	Y	Y	R R	0·08
1 : 16	R	R	R	R	R	R	R	R	YR	YR	R R	0·04
1 : 32	R	R	R	R	R	R	R	R	R	R	R R	0·02
1 : 64	R	R	R	R	R	R	R	R	R	R	R R	0·01
1 : 128	R	R	R	R	R	R	R	R	R	R	R R	0·005
Antitoxin titre of each serum	0·32	0·32	0·32	0·64	2·56	2·56	2·56	2·56	5·12	5·12	0·64	

* Colour in each well: Y = yellow, YR = orange, R = red.

Table 5(a) and (b) shows the colour change patterns of two microplates in which 10 human immune sera were challenged with two different doses of toxin. In Table 5(a) the twofold serum dilutions started at 1 : 64, and went to 1 : 8192 with all wells challenged with a 4 MCD of test toxin. On the other hand, in Table 5(b) the same serum

specimens were diluted twofold from undiluted serum and were challenged with 256 MCD of toxin (64-fold stronger than the ordinary challenge dose). As shown in the two Tables the colour change patterns of the two plates were almost identical and the control titration of the standard antitoxin gave identical titres in terms of antitoxin units by the two plates. Similar titres were also obtained when 64 and 1024 MCD of toxin was used.

These results indicate that a challenge with higher doses of toxin is acceptable for measuring the antitoxin titres of serum whose antitoxin units are expected to be extremely high. By the use of toxin No. 59 the actual antitoxin range detectable was 0·005 to 0·64 iu/ml on a plate challenged with 4 MCD and 0·32 to 40·96 iu/ml with a toxin challenge of 256 MCD [as shown in Tables 5(a) and (b].

Titration of lower level of antitoxin by increasing the volume of serum to be tested

By changing the ratio of toxin and antitoxin in the above procedure it was possible to increase the sensitivity of this technique in order to detect lower levels of antitoxin. Twofold dilutions of the standard antitoxin were made; the wells in the first horizontal row received six drops of each serum dilution whereas those in the second horizontal row received five drops and the number of drops was reduced by one in each subsequent

TABLE 6. Titration of standard diphtheria antitoxin at low levels by the colour change. Effect of increasing the volume of antitoxin

Volume of antitoxin (μl/well)	Antitoxin unit (iu/ml)								
	0·04	0·02	0·01	0·005	0·0025	0·00125	0·00063	0·00031	0·00016
150	Y*	Y	Y	Y	Y	Y	YR	R	R
125	Y	Y	Y	Y	Y	Y	YR	R	R
100	Y	Y	Y	Y	Y	Y	R	R	R
75	Y	Y	Y	Y	Y	YR	R	R	R
50	Y	Y	Y	Y	Y	R	R	R	R
25	Y	Y	Y	Y	R	R	R	R	R

* Colour in each well: Y = yellow, YR = orange, R = red.

row so that one drop was placed in the sixth horizontal row (Table 6). All the wells received diluent to make up a volume of 0·15 ml and were challenged with 4 MCD in 25 μl of the toxin. After incubation at 37 °C for one hour 25 μl of two times concentrated VERO cells were added and the plates incubated. The results are shown in Table 6 from which it appears that a reciprocal end point of neutralization was obtained 0·005 iu/ml in the case of 25 μl (one drop) of serum, 0·00125 iu/ml with 100 μl (four drops) and 0·00063 iu/ml with 125 μl (five drops) of serum. The experimental results were in agreement with the theoretical calculations of the expected values.

Antitoxin titres determined with a purified toxin

Although the effect of diphtheria toxin on cultured cells is well known the possibility still exists that the cell degeneration resulting in the colour change in cell culture medium may be caused by factors other than the toxin. It was considered important, therefore, to confirm whether the result of neutralization reaction with the crude toxins presents

the true diphtheria toxin–antitoxin reaction. The antitoxin titres obtained with highly purified toxin were compared with those determined with the working toxin preparation, No. M-46 (Fig. 3). Sera from 24 children and 12 guinea-pigs immunized with diphtheria

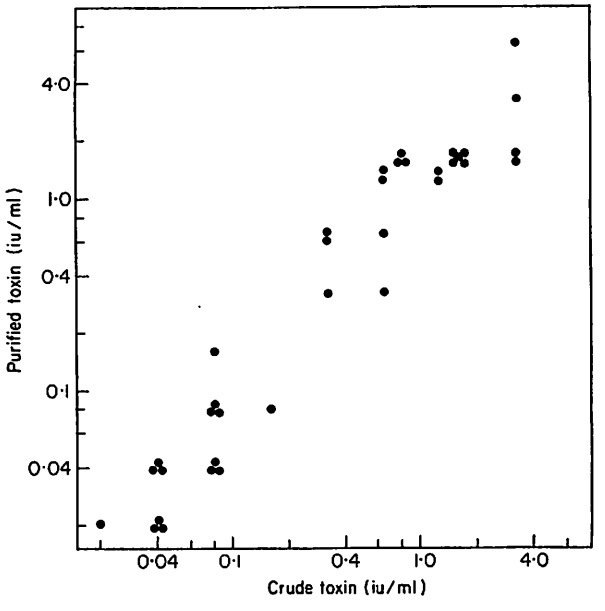

Fig. 3. Correlation between the results of antitoxin titration with purified and crude toxin preparations.

toxoid were titrated by the micro cell culture method challenging in parallel with 4 MCD of each of the two toxin preparations. Titration with both toxin preparations gave very similar results. The statistical analysis of the data gave $b = 1{\cdot}083$ and $r = 0{\cdot}962$. Thus, it was clearly confirmed that the toxin itself is responsible for the CPE and the colour change and that the crude diphtheria toxins may be used for the routine titration of diphtheria antitoxin.

DISCUSSION

The pH colour change method, which had been developed by Salk, Youngner & Ward (1954) for infectivity titration of polio-virus in dispersed cell cultures, was applied for diphtheria toxin and antitoxin titrations by Placido-Sousa & Evans (1957). Since then several authors have confirmed that the cell culture method was as sensitive as the animal skin test and was much more economical. Nevertheless, little progress has been made in its practical application. The reasons for this may be (1) the animal skin test has been well standardized and as it has been considered most reliable for a long time, most microbiologists have paid little attention to an alternative method; (2) the cell culture method was cumbersome for microbiologists who were not familiar with tissue culture techniques and instruments and (3) the experimental conditions which are necessary for its routine use were poorly understood.

We have succeeded in developing a highly sensitive and reproducible micro cell culture method for diphtheria toxin and antitoxin titrations using a minimum amount

of serum by the introduction of the micro cell culture method which had been developed for virology. In this first report, we have explored the level of antitoxin detectable by this method and have investigated the fundamental factors influencing the test particularly when it is used routinely.

In the antitoxin titration we have confirmed that the minimum detectable antitoxin level is dependent upon the quantity of toxoid present in the challenge toxin preparation; that is, the lesser the toxoid content the lower the level of antitoxin detectable. The minimum detectable level of antitoxin was 0·005 iu/ml when 4 MCD of No. 59 and No. M-46 diphtheria toxin were used. These toxins had been stabilized by long storage and had been used routinely in the animal skin test in our institute. The minimum detectable level of antitoxin, however, could be lowered to 0·00125 iu/ml when 4 MCD of a freshly prepared diphtheria toxin preparation such as No. 223 was used. It is considered desirable, therefore, to use a fresh toxin in order to titrate lower levels of diphtheria antitoxin precisely.

We have described two procedures which enable us to obtain detectable levels of antitoxin even lower than 0·00125 iu/ml. This may be done either by using challenge toxin levels lower than 4 MCD or by increasing the quantity of serum to 0·125 ml. In this way one-fifth of the previous lowest level may be detected.

The same antitoxin titres of the test specimens have been obtained irrespective of the concentration of toxin–antitoxin mixture, provided that the neutralization is carried out within the range of a straight line of the neutralization curve. Not only is this so with the standard antitoxin but also with human sera taken after vaccination and identical antitoxin titres were obtained with different doses of toxin challenged. In practice if a serum to be tested is expected to have a high antitoxin titre or if it is entirely unknown, it is possible to titrate the serum against wide ranges of antitoxin titres by making serial dilutions on two transfer plates simultaneously, one of which is challenged with 4 MCD and the other with 512 MCD of the test toxin. If we consider that 0·005 iu/ml of antitoxin is neutralized by 4 MCD of an aged toxin then antitoxin titres ranging from 0·005 to 81·92 iu/ml may be measured simultaneously, using two plates and titres ranging from 0·00125 to 20·48 iu/ml may be measured when fresh toxin is used.

We are of the opinion that the use of transfer plates is essential for the micro cell culture method, because the flat-bottomed microplates are not suitable for diluting small quantities of fluids and also because its bottom is easily scratched with the diluter and this may disturb the formation of a cell sheet. However, the transfer plate may be omitted in our simultaneous cell-seeding method if a round-bottomed plate, which is not toxic to the growth of the cells and does not elute material affecting the pH of the fluid, is available.

The method in which toxin or toxin–antitoxin mixtures are added to preformed cell sheets is also as acceptable as the present method. The simultaneous cell-seeding method described here, however, is not only time-saving but also has an advantage in that the incubation may be made by using pressure-sensitive film eliminating the need for a CO_2 incubator.

Finally, it is emphasized that calf serum used for growing the cells often contains antitoxin activity, and this point must be checked before using the serum particularly if titration of a low titre of antitoxin is required.

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Vero Response to a Cytotoxin of *Escherichia coli*

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A cytotoxin was found in culture filtrates of a number of *Escherichia coli* strains that differed from the known heat-stable and heat-labile enterotoxins of *E. coli*. It was cytotoxic for Vero but not for Y-1 or CHO cells, and its effect on Vero was distinctly different from that of heat-labile enterotoxin. It was labile to heat and antigenically different from heat-labile enterotoxin, and membrane filtration indicated a molecular weight of 10,000 to 30,000.

Recently we described the effect of *Escherichia coli* heat-labile enterotoxin (LT) on the continuous Vero cell line (6). The response of these cells compared favorably with that of Y-1 (mouse adrenal) and CHO (Chinese hamster ovary) cells and of rabbit ileal loops. Nontoxigenic *E. coli* strains showed no response when culture filtrates were tested in any of the above systems.

Some strains of *E. coli* induced a distinctive cytotoxic effect on Vero that was different and easily distinguished from that produced by LT. This report describes the production and properties of this toxin referred to here as VT (toxic to Vero cells).

MATERIALS AND METHODS

Toxin production. *E. coli* enterotoxigenic human or porcine strains, H10401, H10407, B7A, and B2C were obtained from H. L. DuPont (Houston, Tex.); P155, P307, 339, E57, P16, and 711(P307) were from C. Gyles (Canada); SSU 3496, SSU 3515, and M80 31 M1 came from I. K. Wachsmuth (Atlanta, Ga.); and O124, an invasive strain (4), was obtained from C. Park (this laboratory). Strains isolated from infants with diarrhea, H19, H30, and H.I. 1 to H.I. 10, were obtained from C. Gyles, and HW 1 to HW 7 and HSC 1 to HSC 15 came from H. Farkas-Himsley (Canada). Nontoxigenic strains 711 and K-12 were obtained from C. Gyles and V. N. Iyer (Canada), respectively. Eighty-six *E. coli* food isolates were supplied by C. Park and E. Todd (this laboratory). Table 1 lists further details of the strains used.

Erlenmeyer flasks, 250 ml, containing 20 ml of Trypticase soy broth were inoculated and mechanically shaken at 37°C. After 20 to 24 h, the cultures were centrifuged at 17,000 × *g* for 30 min. The supernatants were filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.) and stored at 4°C until assayed. Filtrate dilutions were made in phosphate-buffered saline (PBS), pH 7.0.

Cell culture assay. Stocks of Y-1, CHO, and Vero cells, purchased from the American Type Culture Collection, Rockville, Md., were passaged by trypsinization and grown as monolayers at 36°C in a 5% CO_2 atmosphere. Y-1 cells were grown in Ham nutrient mixture F10 (Connaught Laboratories Ltd., Toronto), CHO in Ham nutrient mixture F12 (Grand Island Biological Co., Grand Island, N.Y.), and Vero in medium 199 with Earle salts (Connaught); all media were supplemented with 10% fetal calf serum (GIBCO). Maintenance of the cell cultures was described elsewhere (6).

Toxin activity was assayed in plastic dishes (Falcon 3008) containing 24 16-mm diameter wells with 0.5 ml of cell culture in each well. Y-1 monolayers were obtained by seeding 10^5 cells per well 3 to 4 days before use; the growth medium was changed at the time of assay. CHO cells, 10^4 in medium F12 without serum, were seeded in each well at the time of assay. For Vero monolayers, 10^5 cells in growth medium were seeded in each well 1 to 2 days before use; the medium was not changed at the time of assay. (The LT effect on Vero monolayers was more defined when growth medium was replaced with PBS just before addition of bacterial filtrates (6). Although the VT effect was more advanced in the presence of PBS than in growth medium after 24 h, Vero cells could not be maintained in PBS for the 4 days necessary to quantitate the toxin by end-point determination.)

To 0.5 ml of cell culture, 0.05 ml of bacterial filtrate was added. For controls, cultures received PBS or Tripticase soy broth. Cultures were incubated at 36°C; the incubation periods are indicated below. Morphological effects were recorded as 1, 2, 3, and 4, these ratings corresponding to roughly ≤25, 50, 75, or ≥90% of cells affected.

Rabbit ileal loop assay. The ileal loop assay was performed in duplicate or triplicate in 9-week-old rabbits by the method of Kasai and Burrows (2). Two milliliters of filtrate or sterile medium was used for each 10-cm loop; after 18 h, the fluid accumulation was measured and expressed as milliliters of fluid per centimeter of gut. A ratio of 1.0 or more was considered positive for LT.

Infant mouse test. Culture filtrates (0.1 ml) mixed with Evans blue dye were injected orally into 1- to 3-day-old mice and incubated for 4 h at 22°C (7). A strain was considered positive for heat-stable toxin (ST) if the ratio of combined weight of the intestines of four mice to the combined weight of the bodies was over 0.09 (1).

TABLE 1. *Characterization of the E. coli isolates examined*

Strain	Serotype	Source	Disease	Enteropathogenic status[a]
H10401 and H10407	O78:K2	Humans[b]	Diarrhea	LT and ST
B7A	O148:H28			
B2C	O6:H16			
339	O15:H11			
SSU 3496 and 3515	O6:K15:H16			
M80 31 M1	—[c]			ST, no LT
O124	O124:K27(B17)	Cheese	Diarrhea in humans	Invasive
P155	O149:K91, 88a, c	Young pigs	Diarrhea	LT and ST
P307	O8:K87, 88a, b			
P16	O9:K9			ST, no LT
E57	O138:K81			
711(P307)	O18:K?[d]	Laboratory derived		LT and ST
711	O18ab:K?:H14	Fowl	Bacteremia	No LT, no ST
K-12	Not typeable	—	—	
H19 and H30	O26	Human infants	Diarrhea	Little or no response in rabbit leal loops
H.I. 1, 8, and 9	O128:B12			
H.I. 2, 3, and 10	O55:B5			
H.I. 4 and 5	O111:B4			
H.I. 6	O18:B21			
H.I. 7	O125:B15			
HW 1	O26:B6			—
HW 2, 3, and 5	O126:B16			
HW 4	O125:B15			
HW 6	O126:B16?			
HW 7	O125:B15?			
HSC 1, 2, 3, 7, 8, 11, 12, and 14	—			
HSC 4 and 5	O55:B5			
HSC 6	O111:B4			
HSC 9	O125:B15			
HSC 10	O126:B16			
HSC 13	O127:B8			
HSC 15	O128:B12			
64 isolates	—	Cheese, one meat	None	
183	O68:H12	Cheese	None	
185	O26:K60(36):H32			
20 isolates	—	Various foods	Diarrhea in humans	

[a] Prior to present study.
[b] Not infants.
[c] —, No information.
[d] Unknown.

Fractionation of bacterial filtrates. A 200-ml pool of bacterial filtrate of strain H30 was fractionated by filtration, in series, through 76-mm diameter Diaflo membranes, XM300, XM100A, PM30, UM10, UM2, and UM05 (Amicon Corp., Lexington, Mass.), which restrict macromolecules in excess of molecular weight 300,000, 100,000, 30,000, 10,000, 1,000, and 500, respectively. Ten milliliters of filtrate from each membrane was retained for assay; the remaining fluid was filtered through the next grade of membrane. The 5- to 10-ml retentate of each membrane was made up to the starting volume with PBS before assay.

Preparation and testing of antisera. Adult albino rabbits were pre-bled and then given, at 7-day intervals, seven sequential intramuscular injections of 1 ml of concentrated culture filtrates from freeze-dried preparations of *E. coli* H10407 or H30. The first injection consisted of a seven times concentration of filtrate emulsified with an equal volume of incomplete Freund adjuvant. The remaining doses were given as a 7 or 10 times concentration without adjuvant. For neutralization studies, culture filtrates or dilutions in PBS were mixed with equal volumes of serial fourfold dilutions of rabbit sera in PBS. Mixtures were incubated for 1 h at ambient temperature (about 22°C) before assaying on Y-1 cells for LT or on Vero cells for VT.

RESULTS

Culture filtrates of 10 out of 136 *E. coli* strains induced a cytotoxic response in Vero cells. Eight of these were associated with diarrhea, seven in human infants and one in a weanling pig; two were isolates from cheese not implicated in disease. Microscopically, the VT-affected cells ap-

peared round but shriveled; many floated free in the medium (Fig. 1). Undiluted culture filtrates induced the above effect in at least 50% of the monolayer within 24 h, but the cytopathic effect advanced with time; maximum titers were obtained in 4 days (Table 2) and varied, with the strain, from 16 to 1,024 (Table 3).

The VT response was readily distinguished from that of LT in Vero cells. With LT, the cells were enlarged, thick-walled, refractile, and possessed several filamentous tendrils (Fig. 2) (6). If the assay of LT was performed in medium 199 with 10% serum, the effect on the cells faded with time; after 3 days, the monolayers appeared

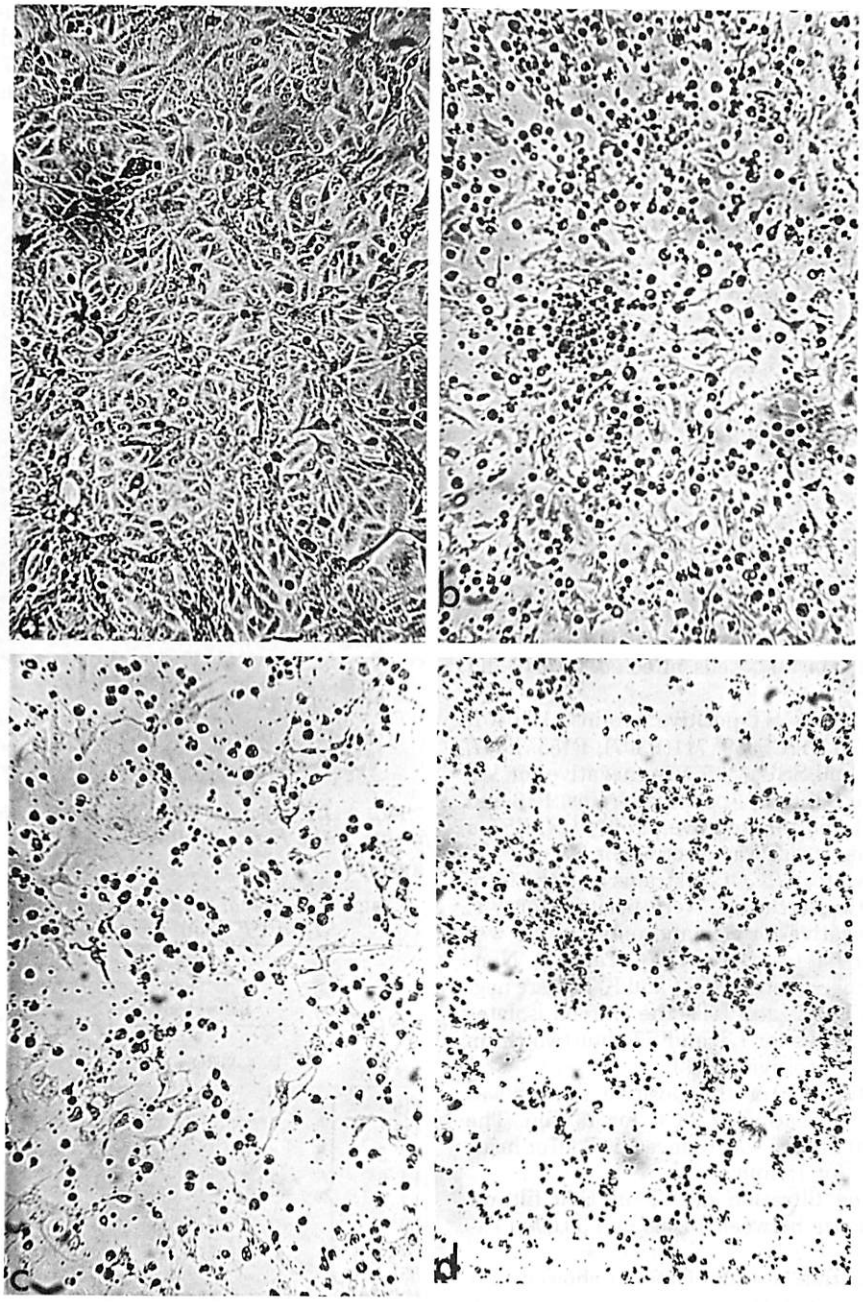

FIG. 1. *VT effect on Vero monolayers: medium control, 1 day (a); with H30 filtrate, 1 day (b), 2 days (c), and 5 days (d). Control monolayers appeared unchanged over 8 days. ×237.*

TABLE 2. *Time course of VT effect of E. coli H19 filtrate on Vero monolayers*

Dilution in PBS of culture filtrate	Day postinoculation					
	1	2	3	4	5	8
Undiluted	4[a]	4	4	4	4	4
1/4	2	3	3	4	4	4
1/16	1	2	3	4	4	4
1/64	—	1	2	3	3	3
1/256	—	—	1	2	2	2
1/1,024	—	—	—	1	1	1
1/4,096	—	—	—	—	—	—

[a] Code: —, no response; 1, ≤25%; 2, about 50%; 3, about 75%; and 4, ≥90% of cells affected.

TABLE 3. *Titer of VT-positive E. coli strains, in Vero monolayers, 4 days postinoculation*

Strain	Source	Disease	Reciprocal of highest dilution inducing morphological change
H19	Human infants	Diarrhea	1,024
H30			1,024
H.I. 5			1,024
H.I. 6			16
H.I. 8			64
HW 1			256
HSC 10			256
E57	Weanling pig	Diarrhea	64
183	Cheese	None	64
185			64

normal. In contrast, cells affected by VT did not recover.

The LT- and ST-positive strains, H10401, H10407, B7A, B2C, 339, 711(P307), P155, P307, SSU 3496, and SSU 3515 were negative for VT as were the ST (only)-positive strains, P16 and M80 31 M1 and the invasive strain 0124. E57, a weanling-pig strain was positive for ST and VT but negative for LT. Of 34 strains isolated from infants with diarrhea, 7 were positive for VT, all were negative for ST, and only HSC10 was positive for LT (as well as VT) (Table 4). Non-enteropathogenic strains 711 and K-12 were negative for VT, ST, and LT. The 86 food isolates were all negative for LT and ST, but two (from cheese) were positive for VT.

VT in filtrates of the positive strains was destroyed by heating at 98°C for 15 min. The titer of H30 filtrate was reduced 50% after heating at 65°C for 15 min.

Membrane filtration of VT in H30 filtrate indicated a size between 10,000 and 30,000 (Table 5).

Neutralization results (Table 6) showed that antiserum against H30 filtrate neutralized VT of all strains except H.I. 8 and E57. Antiserum against H10407 filtrate did not neutralize LT of HSC10. There was no cross-neutralization between LT of H10407 and VT of H30 when different culture filtrates and antisera from three rabbits were used.

DISCUSSION

In general, *E. coli* strains that produced LT did not produce VT. An exception was strain HSC10, but its LT was antigenically different from that of the classical strain H10407. In addition, VT of strains H.I. 8 and E57 were anti-

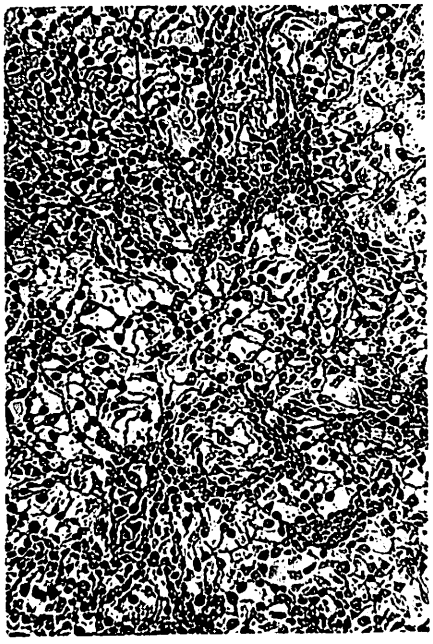

FIG. 2. *LT effect on Vero monolayer with H10407 filtrate, 1 day. For control see Fig. 1a.* ×237.

TABLE 4. *Assays of enterotoxic activity (LT and ST) in VT-positive E. coli strains*

Strain	Enterotoxic activity			
	LT response in:			ST response in infant mouse
	Y-1	CHO	Rabbit ileal loop	
H19, H30, H.I. 5, H.I. 6, H.I. 8, HW 1, 183, 185	−	−	−	−
HSC 10	+	+	+	−
E57	−	−	−	+

TABLE 5. *Fractionation of VT of H30 culture filtrate by membrane filtration*

Membrane size	Reciprocal of highest dilution inducing morphological change in Vero cells	
	Filtrate	Retentate
Control (not filtered)	1,024	
XM300	1,024	0
XM100A	1,024	4
PM30	1,024	64
UM10	64	256
UM2	4	16
UM05	1	0

TABLE 6. *Neutralization of cell response to E. coli toxins by rabbit antibodies*

Strain	Reciprocal of culture filtrate dilution[a]	Cell type for test	Reciprocal of highest dilution to neutralize with antiserum to:	
			H30	H10407
H19	100	Vero	64	0
H30	100	Vero	64	0
H.I. 5	100	Vero	64	0
H.I. 6	1	Vero	64	0
H.I. 8	4	Vero	0	0
HW 1	16	Vero	64	0
183	4	Vero	64	0
185	4	Vero	64	0
HSC 10	1	Y-1	0	0
HSC 10	16	Vero	64	0
E57	4	Vero	0	0
H10407	1	Y-1	0	256

[a] Eight to 16 times the amount that causes toxic effect in cell culture.

genically different from VT of the other eight strains.

The VT titers were generally higher than those of LT in Y-1 or Vero cells (6). As indicated by membrane separation, VT of H30 was a smaller molecule than LT of H10407, i.e., 10,000 to 30,000 as compared to 30,000 to 300,000 (6). Both toxins were destroyed by heating at 98°C for 15 min.

Since the Vero cells failed to recover, the effect of VT was apparently cytotoxic; in totally involved monolayers, many of the cells lifted from the surface. This is in contrast to the cytotonic effect of LT (3). Preliminary studies (not reported here) indicated that in contrast to LT, VT did not stimulate cyclic adenosine 5'-monophosphate production in Vero cells.

It is apparent that several different *E. coli* exotoxins are produced, and they can be measured in cell cultures. The newest of these, VT, may contribute to diarrheal disease in human infants and possibly young pigs. It would be expected that fluid accumulation would result as a cytotoxic response by intestinal cells rather than by the stimulation of cyclic adenosine 5'-monophosphate as with LT. Recently, we noted that the human amnion cell line, FL, was also sensitive to VT (unpublished data). In our studies, the rabbit ileum showed little response with culture filtrates of VT-producing strains. However, fluid accumulation, in this host, has been demonstrated by C. Gyles with concentrated lysates of H19 and H30 (personal communication) and by our laboratory with concentrated purified VT of H30 (unpublished data). The presence of VT in 2 out of 66 culture filtrates from food isolates (not implicated in disease) need not detract from the possibility that VT may be involved in disease. In a similar survey of food, Sack et al. (5) found that 8% of the isolates were enterotoxigenic. Further investigation is obviously required to demonstrate the specific role of VT in enteropathogenic disease.

ACKNOWLEDGMENT

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7.

VERO Cells for Poliovirus Vaccine Production

Introduction

Thanks to the introduction of poliovirus vaccine, poliomyelitis is not a major disease in advanced countries; however, there are many children who suffer from this disease in developing countries. There are two types of poliovirus vaccines: the killed Salk-type vaccine and the live Sabin-type vaccine. These were produced using cells derived from primary African green or rhesus monkey kidney cells. The use of continuous cell lines for the vaccine production has been considered for several years because monkeys, now used for preparation of primary cultures, are decreasingly available and cells derived from these cultures are often contaminated with adventitious agents and must be discarded. Although human diploid cells are considered for the vaccine production in some countries, many institutes encountered difficulties in obtaining desired virus yields in these cells. Vero cells have been found to sustain efficient proliferation of polioviruses and have become one of the candidates for the production of poliovirus vaccines. But, only after establishing the following three points, were they put into practical usage: 1) these cells are not tumorigenic, 2) there are no extraneous viruses, and 3) uniform cell lots can be securely produced using large scale cultivation.

The Institut Mérieux in France and the Food and Drug Administration of the United States have conducted a study on the tumorigenicity of Vero cells and have found no problem in low-passage Vero cells (p. 183). After going through these rigorous examinations, Vero cells have established their role in killed poliovirus vaccine production. The Institut Mérieux has successfully developed a killed poliovirus vaccine using Vero cells and the vaccine has been available in France since 1984 (p. 194, 204, 210).

Furthermore, at the proposal of the World Health Organization, the possibility of Vero cell usage as a candidate culture for the production of live poliovirus vaccines is being considered.

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Tumorigenicity of Vero cells*

Inessa S. Levenbook,† John C. Petricciani† and Bennett L. Elisberg†

One of the current criteria for evaluating the acceptability of cell lines for use in vaccine production is lack of tumorigenicity. Vero cells represent an example of a class of cells known as continuous cell lines. They were derived from African green monkey kidney, and their growth properties and culture characteristics have many advantages over other cell substrates for use in vaccine production. We have tested Vero cells for tumorigenicity in nude mice and in a human muscle organ culture system, and found a significant increase in their tumorigenic potential with increasing passage numbers. Cells at passage 232 and higher produced nodules in all nude mice inoculated. Histologically the nodules were well defined, anaplastic tumors, which exhibited some of the characteristics of renal adenocarcinomas. In about 6 to 8 days all of the nodules began to regress. Data were obtained that suggested an immune mechanism was the basis for the regression phenomenon.

INTRODUCTION

The question of the acceptability of continuous cell lines (CCL) for use in producing viral vaccines has been discussed for several years at various international symposia.[1–4] One of the primary characteristics for the acceptability of human diploid cell lines such as WI-38 and MRC-5 was that these cells lack tumorigenic potential. Recently the World Health Organization (WHO) issued revised requirements for inactivated poliomyelitis vaccine production which included a new section on the use of continuous cell lines as a cell substrate.[5]. WHO prescribed the absence of tumorigenicity as one of the necessary criteria for a continuous cell line to be acceptable

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as a substrate. WHO defined a tumorigenic cell line as one which: (1) produces a palpable nodule that increases in diameter over the 21-day observation period, and (2) shows evidence of metastasis in at least some of the animals.

The Vero cell line was established from the kidney of an adult African green monkey[6] and is currently being used for the production of inactivated poliomyelitis vaccine.[7] Testing these cells for tumorigenicity, van Steenis & van Wezel found nodules in many of the inoculated animals which did not increase in size after the first week following injection, and they designated them as non-progressive,[8] while other authors did not observe nodules.[7] The current study was undertaken in an attempt to clarify the tumorigenic potential of Vero cells by assaying their biologic behaviour in two systems: an *in vivo* system using adult nude mice and an *in vitro* human muscle organ culture system.

MATERIALS AND METHODS

Cell lines

Vero cells (Certified Cell Line 81) were obtained from the ATCC (Rockville, Maryland, U.S.A.) at passage 121. The characteristics of the cells are described in the ATCC Catalogue (1983). The cells were maintained in Eagle's minimal essential medium with 10% fetal bovine serum. Cells of 15 passage numbers, from 124 to 268, were used for *in vivo* assays and the cells of seven passage numbers, from 127 to 265, for *in vitro* tumorigenicity experiments. Cultures from our stock of Vero cells never exhibited any spontaneous cytopathology and tests for mycoplasma contamination were negative. To rule out the possibility of contamination of the Vero cells at high passages with other cells, an isoenzyme analysis was performed by the ATCC. Our stock Vero cells at passage 223 were confirmed as African green monkey. Vero cells were subcultured weekly at a ratio of 1:10. Subculturing procedure was as follows. The medium was removed and the monolayer was rinsed with 0·25% trypsin and 0·02% EDTA in Dulbecco's saline without calcium and magnesium. Enough fresh trypsin and EDTA to cover the cell sheet was added and allowed to absorb for 30 to 60 s. The fluid was then decanted and the culture incubated at 37°C until cells were free (15 to 20 min). Ten millilitres of fresh medium was added and aspirated to break up clumps prior to distribution of cells into new flasks. Viable cell count was made using trypan blue, and the cell concentration was adjusted to 10^7 cells/ml.

WiDr (human colon adenocarcinoma) cell line (Certified Cell Line 218) was received from ATCC and used as the positive control. Primary African green monkey kidney (AGMK) cells, obtained from Flow Laboratories (McLean, Va, U.S.A.), served as the negative control.

In vivo *tests*

Groups of female nude mice (athymic Nu/Nu) obtained from Harlan Sprague Dawley[9] were inoculated intramuscularly (i.m.) into the thigh each with 10^7 cells of either different passage levels of Vero or WiDr or AGMK cells in a volume of 0·2 ml. This route of inoculation was chosen on the basis of our data on the advantage of i.m. versus subcutaneous and intracerebral routes of administration of human colon adenocarcinoma cell line WiDr into nude mice.[10] Most experiments presented here were performed in five- to six-week-old mice, although some were in three- to four- and others in eight- to nine-week-old mice (see Table 1). Natural killer (NK) cells are

TABLE 1. A comparison of the development of palpable tumors in nude mice after i.m. inoculation of Vero, WiDr and AGMK cells

Age of mice (weeks)	Cell type	Passage number	No. of mice with tumors/ no. of inoculated mice	Maximal tumor development*			Progression (P) or regression (R)
				Day(s) post inoculation	Maximal dimension (mm) of largest tumor(s)	Mean maximal dimension (mm) of tumors for groups of mice†	
5–6	Vero	124	0/16	—	—	—	—
5–6	Vero	128	0/10	—	—	—	—
5–6	Vero	162	4/16	8	15‖	15‖	n.d.‡
5–6	Vero	168	5/13	8	15‖	12‖	R
5–6	Vero	232	21/21	6	13	9	R
5–6	Vero	234	5/5	6	15	11	R
5–6	Vero	252	10/10	6	9	6	R
5–6	Vero	261	9/9	6–9	10	6	R
5–6	WiDr	72	13/16	10–14	5§	5§	P
5–6	AGMK	1	0/21	—	—	—	—
3–4	Vero	127	0/5	—	—	—	—
3–4	Vero	140	0/5	—	—	—	—
3–4	Vero	263	4/4	6–9	9	9	R
3–4	Vero	265	5/5	4–7	13	6	R
3–4	WiDr	50	5/5	5–8	7§	5§	P
3–4	AGMK	1	0/5	—	—	—	—
8–9	Vero	149	6/10	6–8	7	5	R
8–9	Vero	249	9/9	6	10	7	R
8–9	Vero	254	5/5	5	15	9	R
8–9	Vero	268	10/10	5–6	15‖	13‖	R

* Measurements were taken in live animals, therefore they represent only approximations of real tumor size.
† Mice without tumors were not included in the calculations.
‡ Not defined: animals were killed at the peak of tumor development.
§ Size of tumors found on designated days and not the maximum attained.
‖ In these experiments the cells for inoculation were resuspended in serum-*free* medium.

known to play an important role in host defense against tumor cells. In order to inhibit NK cell activity in four experiments with five- to six-week-old animals, half of the animals inoculated with Vero cells also received rabbit anti-asialo GM_1 (αAGM_1) (Wako Chemicals, U.S.A., Inc., Dallas, Texas U.S.A.). αAGM_1 diluted 1:10 to 1:20 was injected intraperitoneally on days 1, 4, 8 and 12. The nodules in the muscles were measured twice a week during the observation period, and, because the animals were alive, the sizes recorded are considered approximate. Some of the mice were killed at the peak of nodule development (between days 8 and 15), but most of them were observed for three weeks after inoculation. All inoculation sites irrespective of the presence or absence of grossly apparent nodules were examined histologically except those sites inoculated with the Vero cells of passage 124. In addition sections of the lungs were made from all the nodule-bearing mice inoculated with Vero cells of passages 162, 168, 232, 252 and 254. The pieces of tissue were fixed in Bouin's fluid or 10% formalin, embedded in paraffin, and the sections were stained with hematoxylin and eosin.

In vitro *tests*

Human muscle (HM) cultures were established as previously described.[11] HM was used in this work to provide a substrate devoid of immunologic rejection mechanisms found *in vivo*. 10^6 Vero cells as well as the same number of WiDr and AGMK cells were inoculated onto the HM and cultured for three, seven and 14 days at which times the cultures were fixed and processed for histological examination.

RESULTS

In vivo *tests*

The compiled results of replicate experiments are shown in Table 1. WiDr cells produced progressive tumors in the vast majority of the inoculated animals, while primary AGMK cells failed to form any nodules. Histological examination of the sites of AGMK cell inoculation in many cases showed the presence of very small accumulations of epithelial cells with some glandular-like formations [Plate 1(a)]. Vero cells at passage 124, 127, 128 and 140 did not produce palpable nodules but at necropsy small yellowish formations were found at the site of cell inoculation in most of the animals. Vero cells at passages 149, 162 and 168 caused nodules in some of the mice; the cells at passages 232 and higher produced nodules in all the inoculated animals.

Swelling at the site of injection was noticed in some mice when they were examined on the third day, regardless of the type of inoculated cells. Nodule formation and growth after the inoculation of the Vero cells was rapid in all positive mice, although it differed from experiment to experiment. Beginning from passage 149, definite nodules became palpable from day 6 when some reached up to 10–15 mm in maximal dimension. Both the rate of Vero cell growth and the size of the nodules produced by Vero cells were greater than that observed for WiDr cells during the first two weeks post inoculation. After this time WiDr nodules continued to grow while Vero nodules regressed.

Histologically the sites of cell inoculation in mice inoculated with the cells of low passages, which did not develop palpable tumors, demonstrated morphology similar to nodules caused by higher passage Vero cells [e.g. Plate 1(b) and Plate 2(b)]. The

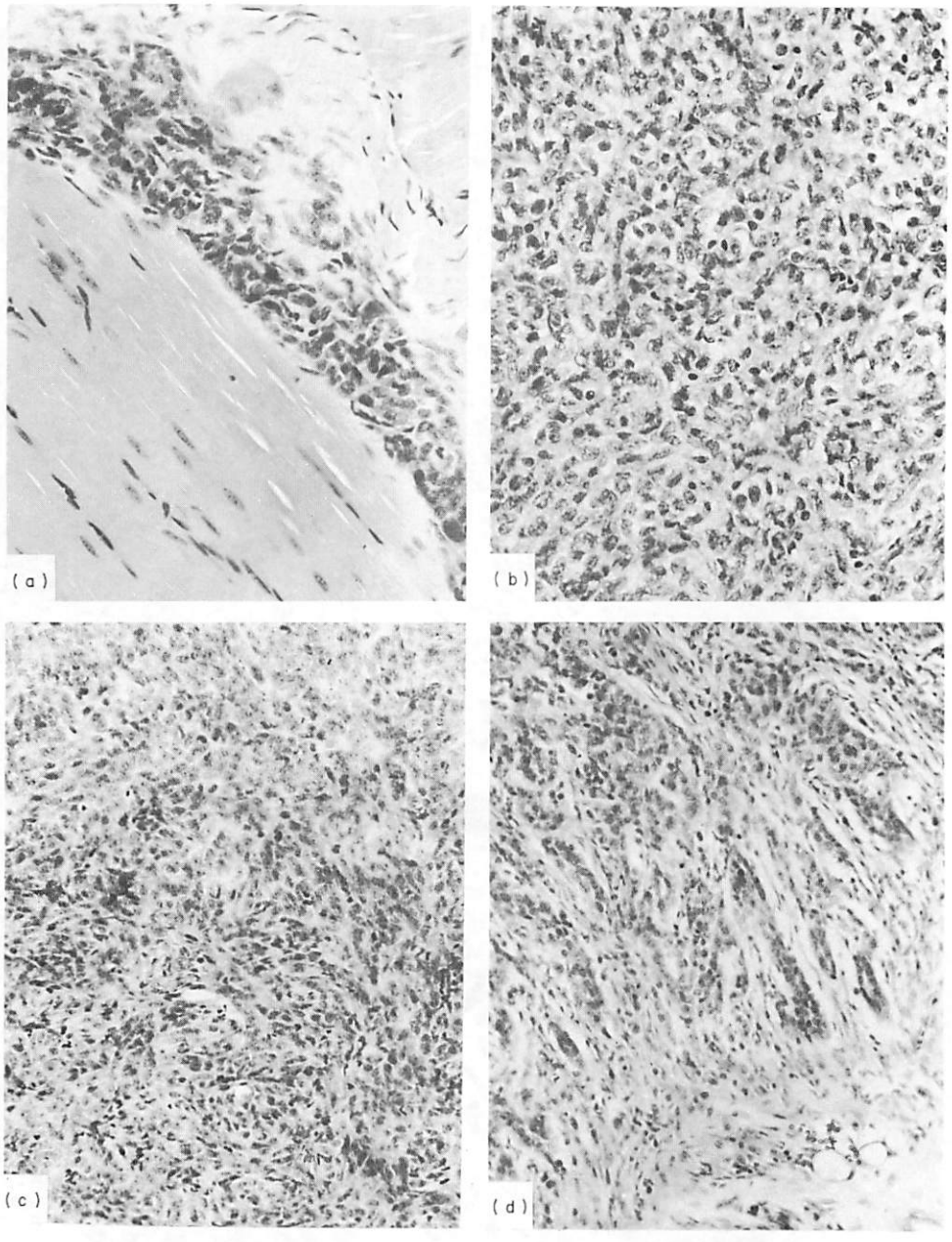

Plate 1. Morphology of the sites of i.m. inoculation into nude mice of primary monkey kidney cells (a), Vero cells passage 128 (b) and small nodules produced by Vero cells passages 149 (c) and 249 (d). Hematoxylin and eosin stained. (a) Non-palpable accumulation of AGMK cells, three weeks after. × 265. (b) Solid arrangement of moderately large cells with 'clear' cytoplasm of some of the cells, three weeks after inoculation of Vero cells passage 128. × 175. (c) Poor histostructural organization of tightly packed cells; stroma is scanty, 10 days after inoculation of Vero cells of passage 249, three weeks after inoculation. Adenomatous structures are distinctive. × 145.

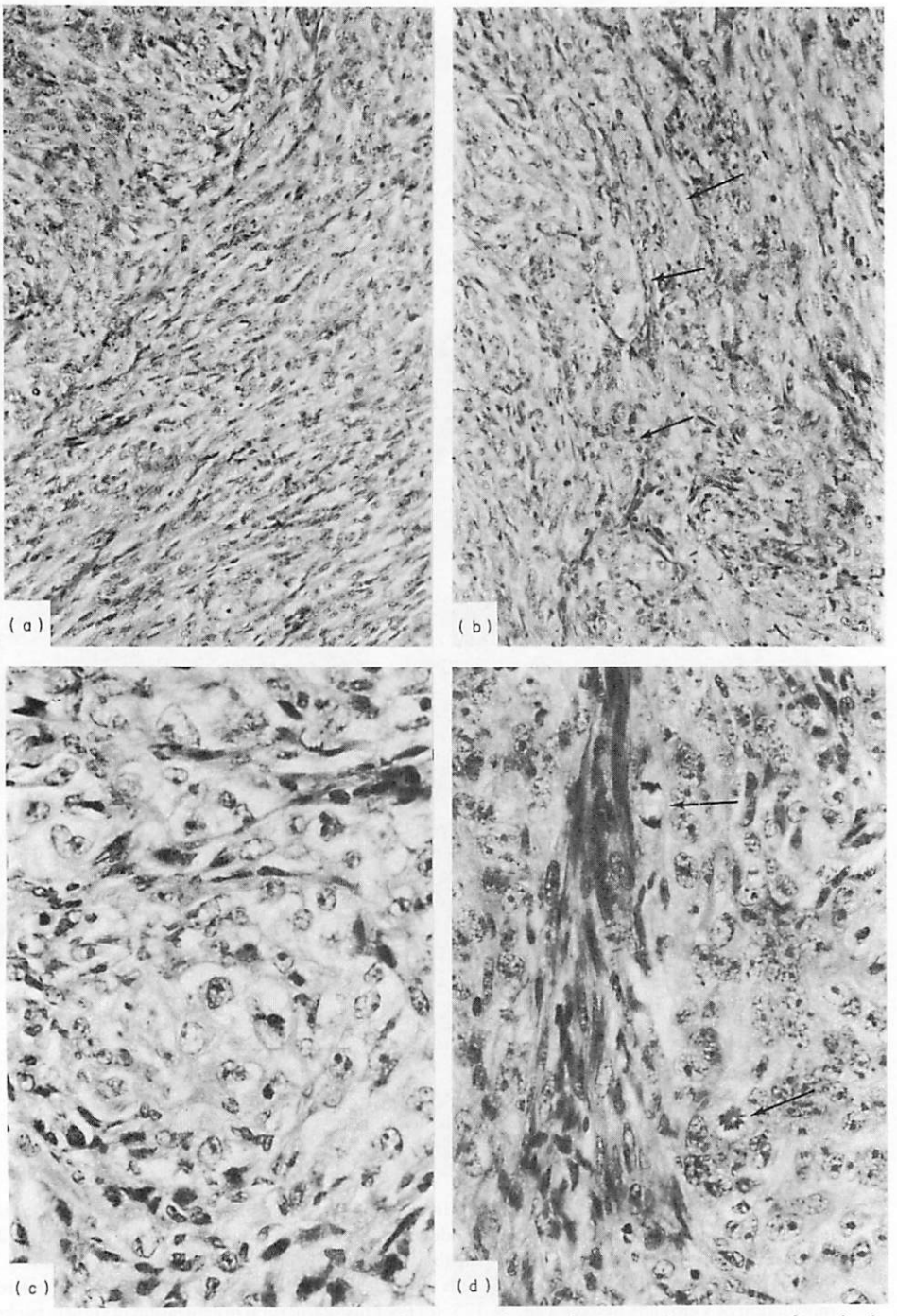

Plate 2. Morphology of large nodules produced in nude mice by Vero cells, passages 162 and 168, eight to 10 days after inoculation. Hematoxylin and eosin stained. (a) Poorly organized (sarcoma-like) pattern of tumor consisting of tightly packed large cells. × 145. (b) Another pattern of the tumor growth. The cells are arranged in solid pseudoalveolar groups surrounded by very thin layers of stroma (arrows). × 145. (c) Detail of the tumor. Rather pleomorphic large tumor cells have vacuolated cytoplasm resembling that of renal carcinoma 'clear' cells. × 435. (d) Detail of the tumor. Mitoses (arrows) are seen among large tumor cells. × 435.

resemblance was especially remarkable with smaller nodules in which glandular-like formations were well-pronounced, predominantly at the periphery [Plate 2(d)]. In central parts of many of the small nodules and in some of the cell accumulations caused by passage 128 one could see solid arrangements of large cells [Plate 1(b)].

Larger nodules, which were observed in the vast majority of animals inoculated with passage 162 and greater, consisted mainly of tightly packed cells randomly arranged in solid patterns or cords, or acini and scanty stroma. The cells were somewhat pleomorphic, mostly large with granular or more often vacuolated 'clear' cytoplasm, vesicular nuclei, well-pronounced nucleoli and some nuclear polymorphism [Plate 2(c)]. Some cells undergoing mitoses were seen [Plate 2(d)]. Large foci of necrosis were frequent. In spite of poor expression of histotypic organization it was possible to find distinctive pseudotubules formed by cells with dark nuclei in many of the sections examined, especially at the periphery of the nodules [Plate 1(d)].

All of the nodules regressed within a short period of time. For example, starting from passage 232 the cells produced large nodules by the sixth day and had regressed markedly by day 14. However, gross examination of the injection site three weeks and later after the inoculation of the cells revealed the remnants of nodules which were microscopically consistent with the histology observed earlier. Lung metastases were not found in any of the mice examined.

As can be seen in Table 1, inoculation of mice of different ages did not show any significant difference in the day(s) of tumor appearance, or in tumor size. The average tumor sizes in individual experiments within each age group differed significantly, as did the tumor size in individual mice in each experiment. However, the impression was gained that in those experiments in which the cells for inoculation were resuspended in serum-free medium they appeared to cause larger nodules.

αAGM_1 seemed to have no effect on the frequency of nodule induction, time of appearance, maximum size attained or degree of regression. In order to determine whether or not the regression of nodules was due to an immune response, an additional experiment was carried out using ten animals which had been inoculated with passage 232 Vero cells. On day 14 after the first inoculation of Vero cells when the nodules had almost regressed the animals received a second i.m. injection of 10^7 cells of passage 234 in the limb not injected previously. Very small nodules (2 and 3 mm in diameter) were detected in only two animals when they were examined at necropsy on day 8. None of the remaining eight mice, which were followed up to 21 days, showed any evidence of nodule formation, and no remnants of Vero cell growth were found macroscopically or microscopically. Five other animals inoculated with the passage 234 cells which had not previously encountered Vero cells, developed large nodules by the sixth day.

In vitro *tests*

AGMK cells grew on the surface of HM as a monolayer of rather monomorphic cells [Plate 3(a)]. WiDr cells manifested an abundant multilayered growth of pleomorphic cells some of which were in mitosis and exhibited a tendency to invade the muscle [Plate 3(b)].

Vero cells of passages 127, 132 and 140 showed growth of epithelial cells in a combination of multi- and monolayered patterns; the cells revealed a tendency to pile up in papillary-like structures [Plate 3(c)]. Vero cells at passage 230, 263 and 265 expressed more advanced growth with multilayered areas predominating and increased cell polymorphism and invasion [Plate 3(d)].

DISCUSSION

There was a significant difference in gross findings between the results of *in vivo* tumorigenicity tests of Vero cells at lower and higher passages. Thus, cells at passages 124–140 (our experiments) produced no palpable nodules. There is information about negative results with the cells of the 134th passage.[7] In our experiments inoculation of nude mice with 10^7 cells of passage level 149 and higher resulted in the occurrence of nodules in some of the animals. Van Steenis & van Wezel[8] inoculated anti-rat thymocyte globulin-treated newborn rats with Vero cells at the 144th passage and described the development of nodules in nine of out ten animals receiving 10^7 cells. Vero cells of the highest passages studied by us (from 232 on) produced nodules in 100% of the nude mice inoculated. The nodules which developed from the cells of passage 232 and higher attained sizes up to 15 mm in maximal dimension in approximately six days.

With respect to the nature of the nodules the following features provide evidence that the nodules represented tumor growth and not the propagation of normal cells: (1) compared to the very limited propagation of primary AGMK cells in nude mice the Vero cells grew progressively during the first week after inoculation; (2) with increased passage numbers of the Vero cells the incidence of the nodules and the rate of their growth were greater; (3) starting from passage level 162 many nodules reached a moderately large size (up to 15 mm in maximal dimension); (4) the histological picture, especially of the larger nodules, was consistent with malignant growth.

Smaller nodules in most cases showed more differentiated tumor growth patterns. The tendency of the cells to form pseudotubular structures indicated that these tumors were adenocarcinomas. The presence of large cells with granular, vacuolated or 'clear' cytoplasm suggested a similarity of these tumors to experimental (e.g. murine) and human renal adenocarcinomas.[12,13] In the HM system the Vero cells also manifested the patterns of malignant growth. Their tendency to form papillary-like structures in the HM system was also consistent with the characteristic of renal epithelial tumors.

Non-invasive local growth of the tumors and the absence of lung metastasis in nude mice examined could not be considered as contradictive to the diagnosis of adenocarcinoma. It is known that in adult nude mice many neoplastic cell lines including HeLa produce well-delineated tumors which are non-metastasizing at least during a three-week observation.[14,15]

The greater tendency towards tumorigenicity of higher passage Vero cells might be due to an increase in the number of tumorigenic cells present in small numbers in the population of Vero cells in lower passages. It appears to be a specific manifestation of the progression of tumorigenicity.[16,17] Cloning both higher and lower passage numbers would be helpful in identifying the existence of such heterogeneous cell populations.

The phenomenon observed with Vero cells of initial malignant growth followed by regular regression has not been reported previously. However, the spontaneous regression of renal cell carcinomas in nude mice following a period of progressive growth was mentioned by Sordat in the discussion following the presentation by Tamaoki *et al.*[18] The mechanism of regression of a primate renal adenocarcinoma transplanted into nude mice deserves special study. Our data suggest an immunologic basis for the tumor regression and the failure of αAGM_1 to suppress regression indicates that natural killer cells might not be involved.

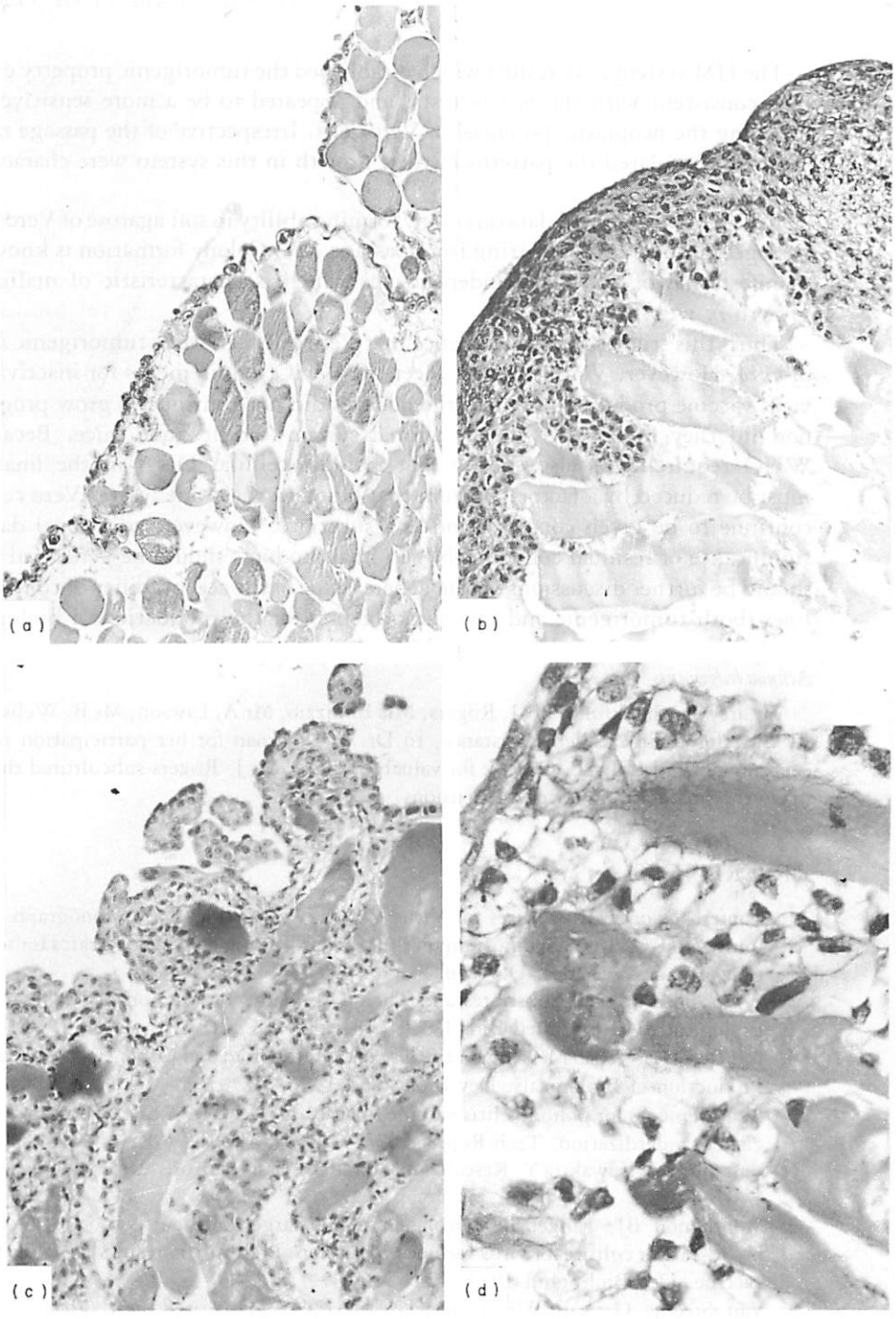

Plate 3. Patterns of growth of primary monkey kidney cells (a), human colon adenocarcinoma cells WiDr (b) and Vero cells [(c) and (d)] on human muscle organ culture, seven days after inoculation. Haemotoxylin and eosin stained. (a) Monolayered growth of AGMK cells on the surface of HM. × 160. (b) Multilayered growth of WiDr cells with some invasion. × 160. (c) Vero cells passage 132. Multilayered growth with papillary-like formations and invasion into the muscle. × 145. (d) Vero cells passage 230. Pleomorphic cells with vacuolated or 'clear' cytoplasm invaded into muscle fibers. × 395.

The HM system gave results which established the tumorigenic property of the Vero cells consistent with the *in vivo* tests, and appeared to be a more sensitive assay for revealing the neoplastic potential of Vero cells. Irrespective of the passage number of the cells inoculated the patterns of their growth in this system were characteristic of tumor.

Recently we obtained data on colony forming ability in soft agarose of Vero cells of all passage numbers tested starting from passage 127. Colony formation is known to be a manifestation of anchorage independence which is characteristic of malignant cell growth *in vitro*.[19]

Thus, this study provided evidence that Vero cells behave as tumorigenic *in vivo* and *in vitro*. However, Vero cells still meet the WHO requirements for inactivated polio virus vaccine production because the nodules did not continue to grow progressively, nor did they metastasize in our animal system—adult nude mice. Because those WHO requirements also specify that residual cellular DNA in the final product must be reduced by a factor 10^8 from that found in the crude lysate, Vero cells should continue to be given consideration as a substrate. However, additional data on the significance of residual cellular DNA in final products should be generated and there should be further discussions on the general issue of the acceptability of continuous cell lines (both tumorigenic and non-tumorigenic) for the production of biologicals.

Acknowledgements

We are very thankful to Mr J. Rogers, Mrs E. Lizzio, Mr A. Lawson, Ms B. Webster and Ms R. Burt for their excellent assistance, to Dr V. Koyfman for her participation in the HM experiments and to Dr E. Gorelik for valuable advice. Mr J. Rogers subcultured the cells and made the preparations of cell suspensions.

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THE LARGE-SCALE CULTIVATION OF VERO CELLS IN MICRO-CARRIER CULTURE FOR VIRUS VACCINE PRODUCTION PRELIMINARY RESULTS FOR KILLED POLIOVIRUS VACCINE

B.J. Montagnon, B. Fanget and A.J. Nicolas

ABSTRACT

As the increasing shortage of monkeys is a reality, the application of an alternative cell substrate for large-scale production of Killed Poliomyelitis Vaccine (KPV) was studied. Through progress of scientific knowledge the non-tumorigenic VERO cell line was considered to be a suitable alternative cell substrate for this purpose. The Master-Cell-Bank and Working-Cell-Banks prepared by us are giving a practically inexhaustible cell source. Using micro-carrier culture, weekly more than 400 billions of cells at a concentration of 10^6 cells per ml could be obtained for virus inoculation. The virus yield per cell was at least as high as for primary monkey kidney cells. Processing of virus harvests could be performed according to the methods used at the production on primary monkey kidney cells. From a technological view-point large-scale production of KPV on VERO cells appears to be possible economically. More research on the safety control might be necessary.

INTRODUCTION

From 1977 to 1979 we have tried to produce Killed Poliovirus Vaccine (KPV) by large-scale cultivation of primary monkey kidney cells (PMKC) in micro-carrier culture according to the methods described by van Wezel et al. (7). From fifty 140 l cultures twenty-three batches of monovalent KPV have been prepared. Later the application of subcultured monkey kidney cells was studied. As the growth rate of Patas monkey kidney cells was lower than of cynomolgus kidney cells (8), the results were disappointing At that time we were encouraged by Jonas Salk to study the application of a non-tumorigenic cell line for which the VERO cell line was chosen. In this paper a short review of this study is given.

MATERIALS AND METHODS

1. *Cells*

From 1 ampoule of VERO cells, obtained from the American Type Culture Collection (ATCC) in May 1979, a Master Cell Bank (MCB) and Working Cell Banks (WCB) were prepared.

2. *Media and culture methods*

Medium 199 in Earle's BSS supplemented with 5% foetal calf serum was used for the preparation of a MCB. The cells were grown in monolayer cultures (TC Falcon 25 and 75 and Roux bottles).

For the preparation of WCB's the cells were subcultivated in Eagle's MEM in Earle's BSS supplemented with 5% new born calf serum and 0.2% lactalbumin hydrolysate in TC Falcon bottles and microcarrier cultures. The same medium has been used for large-scale cultivation in microcarrier culture of production batches.

The poliovirus cultivation was performed in medium 199 in Earle's BSS without serum.

3. *Micro-carrier*

Cytodex 1 (Pharmacia) at a concentration of approximately 1 g/l was used as micro-carrier. The beads were washed with PBS, autoclaved and stored at 4°C. Before use they were washed successively with sterile PBS and tissue culture medium.

4. *Poliovirus strains*

Type 1 Mahoney, type 2 MEF_1 and type 3 Saukett kindly supplied by the Rijksinstituut voor de Volksgezondheid, Bilthoven, were used as seed virus. Master-Seed-Virus and Working-Seed-Virus suspensions were prepared on PMKC.

5. *Poliovirus titrations*

Virus titrations were performed by seeding serial dilutions on roller-tubes of Patas PMKC. The $TCID_{50}$ end-point was calculated by the method of Spearmann and Kärber.

6. *D-antigen determination*

The D-antigen determinations were performed according to a method described (6) using the direct ELISA test.

7. *Processing virus harvests*

Concentration, purification and inactivation of the virus harvests were performed according to methods described (7).

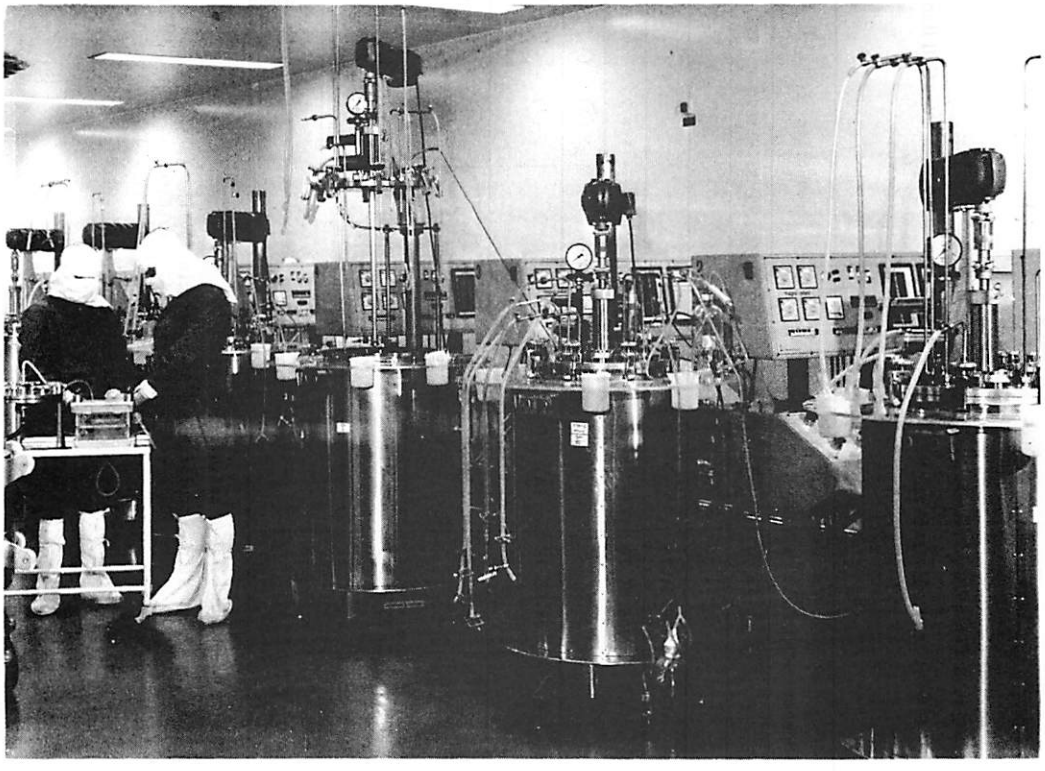

Fig. 1. Fermentors used for large-scale cultivation of cells and virus in micro-carrier culture.

Table I. Master Cell Bank

Day Number	Dates	Production		Surface cm2	Passage Number	# PDL
0	05.22.79	(A) 1 Ampoule VERO (ATCC.CCL 81 - VERO F 1415)			124	
7	05.29.79	(A) 1 F 25		25	125	1
10	06.01.79	(A) 2 F 75		150	126	3
14	06.05.79	2 Roux + 6 F 75		850	127	3
		(A) (2 F 75)	(A) (3 F 75) *	150 - 225		
17	06.08.79	(A) 10 F 75	(B) 15 F 75	750 - 1125	128	2.5
		(4 F 75)	(4 F 75) *	300 - 300		
24	06.15.79	(A) 12 Roux	(B) 12 Roux	2400 - 2400	129	4
		1 R ⟶ REJECTED				
29	06.20.79	11 R ⟶ 111 Ampoules (A) 1.8×10^6 cells				
30	06.21.79		12 R ⟶ 117 Ampoules (B) 2.2×10^6 cells			13.5

(A) Medium without antibiotics

(B) Medium with antibiotics — Streptomycin (sulfate) 75; Polymyxin (sulfate) 14; Néomycin (sulfate) 35 — I.U./1 ml

* Only aliquot TC Falcon (75) used to prepare the next step.

RESULTS

Tables I and II show the details of the preparation of the MCB and WCB starting from one ampoule of VERO cells received from ATCC at the 124th passage. At first the cells were subcultivated in medium without antibiotics. Later on subcultures were split into two lines : line A was further subcultured in medium without antibiotics and line B in medium plus antibiotics in order to avoid risks of complete loss. From both lines cells were frozen at the 129th passage in aliquots of about 1 x 10^6 cells per ampoule. These ampoules are stored in liquid nitrogen as MCB A and B.

One ampoule of MCB B was used for the preparation of WCB 2 (Table II). The cells were subcultivated in monolayer and micro-carrier culture up to the 137th passage, and subsequently frozen in liquid nitrogen in 10 ml and 1 ml ampoules in amounts of 120 x 10^6 cells. Only the 10 ml ampoules coded WCB2 are used.

From the WCB cells were further subcultivated for production purposes as indicated in Table III. According to this schedule potentially 400 billions of VERO cells can be made available for virus inoculation each week. The limiting factor for scaling up is the capacity of the 200 l stainless steel fermentors (Fig. 1). Larger fermentors will be needed for this.

In Figure 2 the growth curve of the VERO cells in micro-carrier culture is shown and Figure 3 shows scanning micrographs of VERO cells on the carriers at 16, 24 and 72 hours after inoculation. From these results and those of the first 7 experiments at production scale (Table IV) it may be concluded that cell growth and virus yields are comparable to those obtained with PMKC.

The controls on VERO cells as proposed in the WHO Revised Requirements for Poliomyelitis Vaccine (Inactivated), 1979, have been performed according to the schedule given in Table V. So far no indication was found for the presence of extraneous agents. Also tests for tumorigenicity of the cells in nude mice appeared to be negative whereas Hep 2 cells were positive at a similar inoculation level of 1 x 10^6 cells per mouse. The test in monkeys (3) is now under investigation. The first experiments failed as the tumoral nodules clinically observed in the monkeys after inoculation with Hep 2 control cells regressed at day 10 and disappeared at day 14, probably because of the fact that the immunosuppression by the antithymic serum was too low.

These tests will be repeated. Further cells were tested for contamination with Mycoplasma both cultivable (10) and non-cultivable (11). All tests were negative.

DISCUSSION

In 1976 (2) it had already been suggested that lymphoblastoid cell lines might be used for production of biological substances under certain conditions. In Lake Placid 1978, (9) the problem of the application of continuous cell lines was discussed more broadly and it was concluded that studies in this direction should be initiated. Because of this and the encouragment of Jonas Salk, application of the non-tunorigenic VERO cell line for large scale production of killed poliomyelitis vaccine was studied. From our results it can eb concluded that large scale production of this vaccine on these cells

Table II. Working Cell Banks Number 2 02.04.80

Day Number	Dates	Production	Surface cm2	Passage Number	# PDL
0	12.13.79	1 Ampoule Master Cell Bank with antibiotics (B)		129	
5	12.10.79	1 F 75	75	130	2
11	12.24.79	1 Roux	200	131	1.5
18	02.31.79	8 Roux	1 600	132	4
22	01.04.80	64 Roux ⟶ 8 R + 56 R ⟶ Assays	12 800	133	4
29	01.11.80	64 R ⟶ 8 R + 56 R ⟶ Assays	12 800	134	4
36	01.18.80	64 R ⟶ 56 R + 8 R ⟶ Assays	12 800	135	4
43	01.23.80	(Microcarrier 1 g/L) 1 x 20 L Fermentor	120 000	136	5
50	01.30.80	(Microcarrier 1 g/L) 1 x 130 L Fermentor	780 000	137	3.25
55	02.04.80	- 105 Ampoules 10 ml (120 x 10^6 cells) WCB n° 2 - 107 Ampoules 1 ml (120 x 10^6 cells) *			27.75

*Not used.

Table III. Flow-sheet of VERO cell culture for poliovirus production

Day Number	Production	Control	Surface cm2	Total cells x 10^6	Passage Number	# PDL
0	WBC n° 2 1 ampoule 10 ml			120	137	
7	1 x 1 L Fermentor (Microcarrier 1 g/L)		6 000	1 000	138	5
13	1 x 5 L Fermentor (Microcarrier 1 g/L)		30 000	5 000	139	2.5
20	1 x 20 L Fermentor (Microcarrier 1 g/L)		120 000	20 000	140	2
27	1 x 130 L Fermentor (Microcarrier 1 g/L)		780 000	130 000	141	3.25
34	140 L 140 L 140 L (Microcarrier 1 g/L) 3 x 140 L	700 x 10^6 cells	2 520 000	420 000	142	1.75
	VIRUS INOCULATION					
37	CRUDE VIRUS HARVEST					14.5

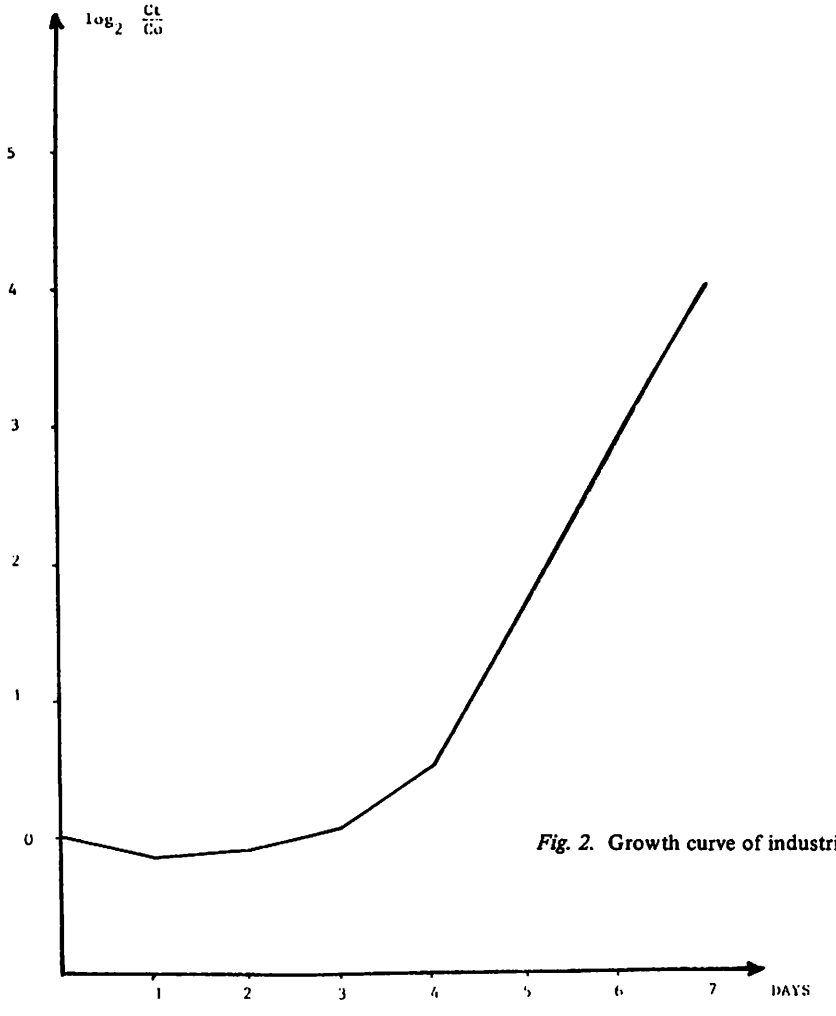

Fig. 2. Growth curve of industrial culture.

grown in micro-carrier culture will be no problem technologically. The main point will be the acceptance of these cells from a safety point of view. So far tested they meet the requirements as formulated in the proposed WHO Revised Requirements for Poliomyelitis Vaccine (Inactivated) 1979. As in the case of human diploid cells it might be necessary for a clear demonstration of the safety of this new cell substrate that eventually a larger range of control tests should be performed. Control tests which could be considered for this purpose are karyotyping, co-cultivation with or fusion with other cells such as MRC_5 cells for control on the absence of virus genomes, tests on the absence of C particles by electron microscopic examination and reversed transcriptase activity determination. Studies on these control tests have been started. Further we are checking whether by our purification process the cellular nucleic acids are eliminated from the virus suspension using the procedure followed by Crainic.

If the application of the non-tumorigenic cell lines like VERO cells is accepted for the production of KPV, it will be possible to produce large amounts of this vaccine with a potency as used in the latest clinical studies (4, 5).

Acknowledgments

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Table IV. Cell and virus growth at an industrial scale

Exp. N°	Preculture			Industrial culture			Virus culture		
	Vol. L.	Cells/ml x 10^3		Vol. L.	Cells/ml x 10^3		Type	Virus yield/ml	
		Start	End		Start	End		D U	Log $TCID_{50}$
1	130	90	1 290	140	302	790	1	78	8.10
				140	302	2 430	1	75	8.30
				140	302	2 300	1	82	8.10
2	130	90	680	140	216	968	2	25	8.20
				140	216	1 180	2	21	8.00
				140	216	1 300	2	23	7.60
3	130	108	1 134	140	245	979	3	67	8.00
				140	245	1 090	3	60	7.50
				140	245	1 070	3	60	7.60
4	130	101	1 443	140	100	1 187	1	98	8.40
				140	100	987	1	84	8.20
				140	100	1 406	1	92	8.40
5	130	88	206 (1)	140	58	1 037	2	18	7.90
				140	58	878	2	12	8.90
				140	58	1 012	2	23	8.50
6	130 *	54	896	140	109	609	3	47	7.30
				140	109	575	3	44	7.30
				140*	109	503	3	59	7.30
7	130 *	83	987	140	239	918	1	ND **	7.60
				140	239	771	1	ND	8.10
				140*	239	878	1	ND	8.10

* Medium without Lactalbumin Hydrolyzate.
(1) Fault on pO_2 regulation.
** ND : not done.

a)

b)

c)

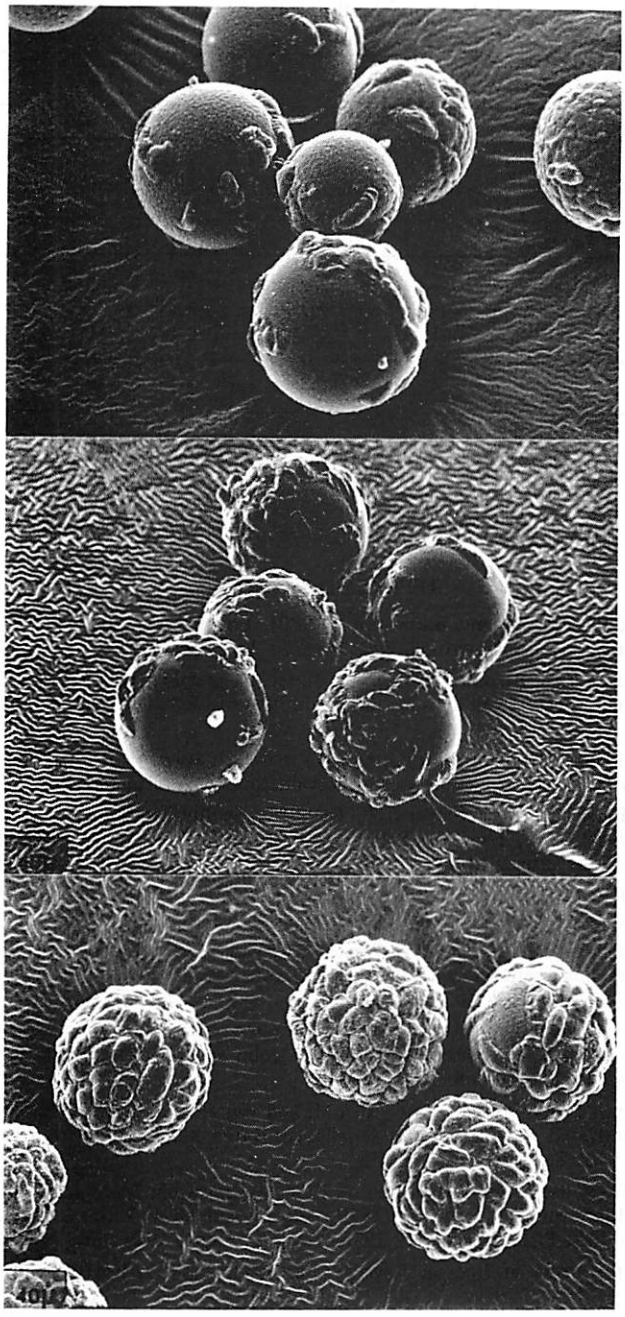

Fig. 3. Scanning micrographs of VERO cells on beads
a) at 16 h b) at 24 h c) at 72 h

Table V. Control cells management

Day Number		Passage Number
27	700×10^6 cells	141
	60×10^5 = 20 ampoules; 105×10^6 = 35 F 75; 500×10^6 = 50 Roux	142
31	12 F (Had.)	
34	Medium change + S_1 sampling → 10 R → Animal eggs } controls	
37	12 F (Had.) Medium change + S_2 sampling (40 R)	
48	Medium change + S_3 sampling (40 R) + Had. (24 R)	
	11 F { Caryology; Non-tumorigenicity	

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DISCUSSION

MELNICK : Can you tell us what the appropriate cost of the vaccine will be when it is prepared in fermentors ?

VAN WEZEL : In view of the new developments going on it is difficult to give an exact price. However, we expect that when the production is brought into an industrial scale using non-tumorigenic cell lines the price will drop automatically.

5th General Meeting of ESACT, Copenhagen, Denmark, 1982.
Develop. biol. Standard. Vol. 55, pp. 37-42 (S. Karger, Basel, 1984)

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THOUSAND LITRE SCALE MICROCARRIER CULTURE OF VERO CELLS FOR KILLED POLIO VIRUS VACCINE. PROMISING RESULTS

B. Montagnon, J.C. Vincent-Falquet and B. Fanget

ABSTRACT

Through the progress of scientific knowledge the Vero cell line was considered to be a suitable alternative cell substrate for the industrial production of Polio Virus.

Using microcarrier culture, more than 10^{12} cells could be obtained weekly for virus inoculation.

The virus yield is around 60 D units/ml for type I ; 20 D units/ml for type II, and 50 D units/ml for type III.

INTRODUCTION

At the Symposium on Reassessment of Inactivated Poliomyelitis vaccine held in Bilthoven in June 1980, we presented our preliminary results for killed poliovirus vaccine prepared from the large scale cultivation of Vero cells in microcarrier culture.

On the industrial scale, we used the 200 litre tanks and three separate cultures of 140 litre gave us the medium culture for the poliovirus cultivations.

Recently, we scaled up to the 1000 litre scale for the Vero cells final step culture and poliovirus production.

Now we present the promising preliminary results.

MATERIALS AND METHODS

Cells, media, microcarriers and poliovirus are the same as described in 1980 (1). Briefly, the Vero cells line received from ATCC (CCL-81) at 124th passage was prepared for Master Cell Bank at the 129th passage.

The Working Cell Bank (WCB-2) combining monolayer and microcarrier culture was prepared at the 137th passage.

For large scale production the cells are at the 142nd passage through five steps, A, B, C, D, E, scaling up from 1 litre vessel to 1000 litre tanks.

At step D, a sample of trypsinized cells corresponding to 500 ml of final culture is cultivated in 20 Roux bottles for control cells.

The 200 litre vessels are the Bilthoven Unit Magno-Paljas (Contact – the Netherlands) and the 1000 litre units were developed in our Institute. The management of culture is conducted by automated microprocessor device (Fig. 1).

The microcarrier is Cytodex-1 prepared and sterilized as recommended by Pharmacia. The concentration generally used is 1.5 g/litre.

The poliovirus type 1 Mahoney, type 2 MEF-1 and type 3 Saukett have been prepared by cultivation on Vero Cells for the virus seed.

Virus suspensions are clarified and filtrated at 0.2 micron, concentrated by ultrafiltration and purified in two steps through chromatographic procedures : gel filtration on Sepha-

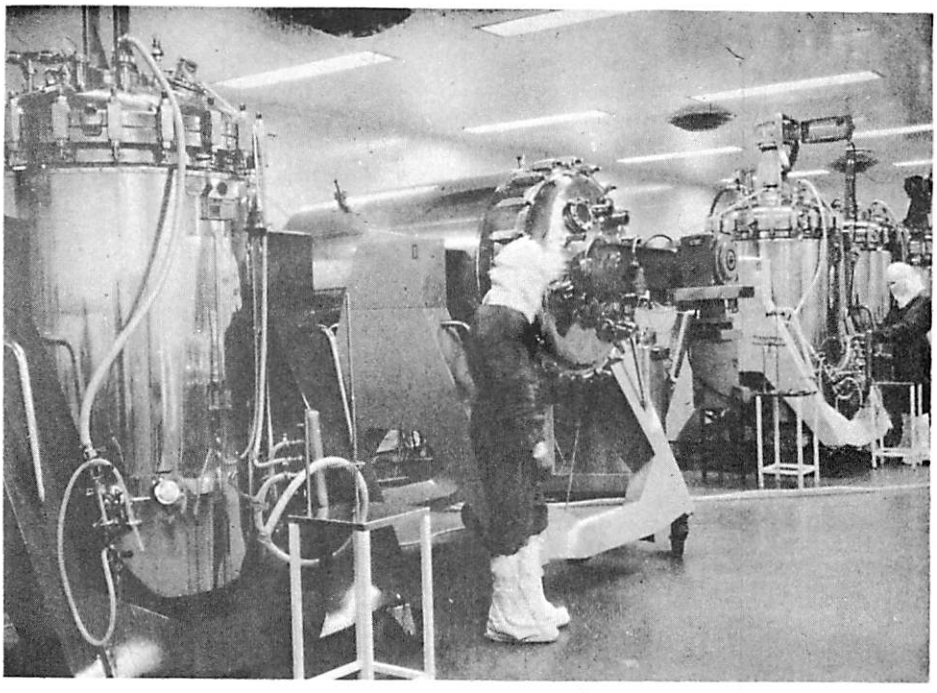

Fig. 1. 1000 L. units used for industrial scale production of killed poliovirus vaccine.

rose CL-6 B (Pharmacia) and ion-exchange on DEAE- dextran-Spherosil (Spherodex) developed by Institut Mérieux (2).

The inactivation is performed by 1 : 4000 formalin at 37° C for 12 days, as classically performed for the SALK polio vaccine.

RESULTS

We have already prepared several cultures in 1000 litre tanks. Generally the cell input was multiplied by a factor ten, in six to seven days.

Our best score was obtained with 1 g/L of beads : starting culture at $10^{5.2}$ cells/ml we obtained $10^{6.2}$ cells/ml after five days. The cell growth curve is as shown (fig. 2).

The crude virus yield was an average of 60 D units/ml for type 1, 20 D units/ml for type 2 and 50 D units/ml for type 3, which are medium values.

Now we intend to improve the virus production.

DISCUSSION

At the Bilthoven Meeting in 1980, we discussed the ability of the Vero cell line as a good candidate for the production of killed poliovirus vaccine (KPV) on a large scale.

All the tests performed on the Vero cell line have given a consistent answer. Particularly, the tests of tumorigenicity on newborn rats as described by Van Stee-

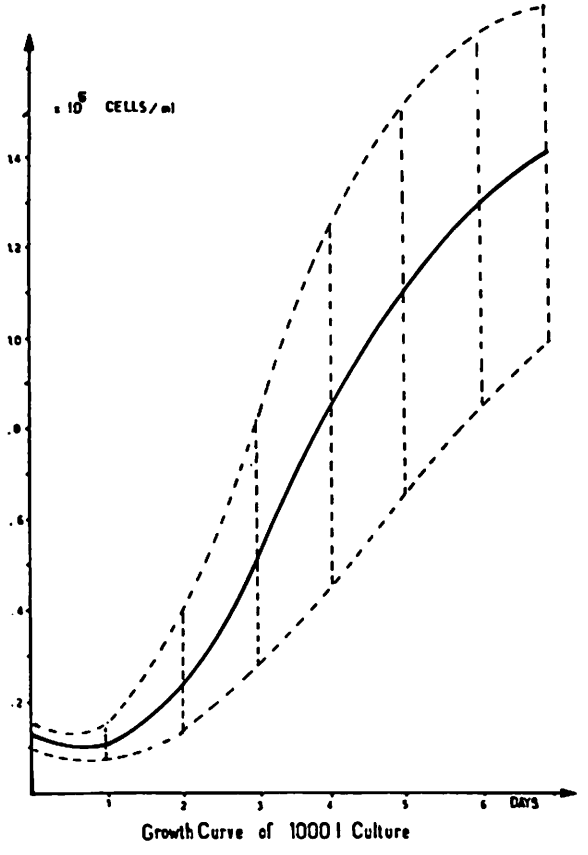

Fig. 2. Vero cell growth curve on microcarrier system.

nis (3) were constantly negative with Vero cells and positive (plus metastasis) with HeLa cells.

The karyologic analyses performed at three steps have shown quite a good homogeneity of the number of chromosomes (Fig. 3). The figures obtained for A, B and IM cells correspond :

- A to passage 131 coming directly from the master cell bank.
- B to these cells after five more passages on microcarrier culture.
- and IM are Vero cells from the working cell bank (WCB-2) plus ten passages on microcarrier culture.

These diagrams are quite different from those obtained from the literature (Fig. 4) : but the number of metaphases counted by our colleague Dr. Jeanine Patet, was more than double the count used by the other authors. From the cytogenetic studies, the Vero cells grown on microcarrier are similar to the Vero cells of our original stock.

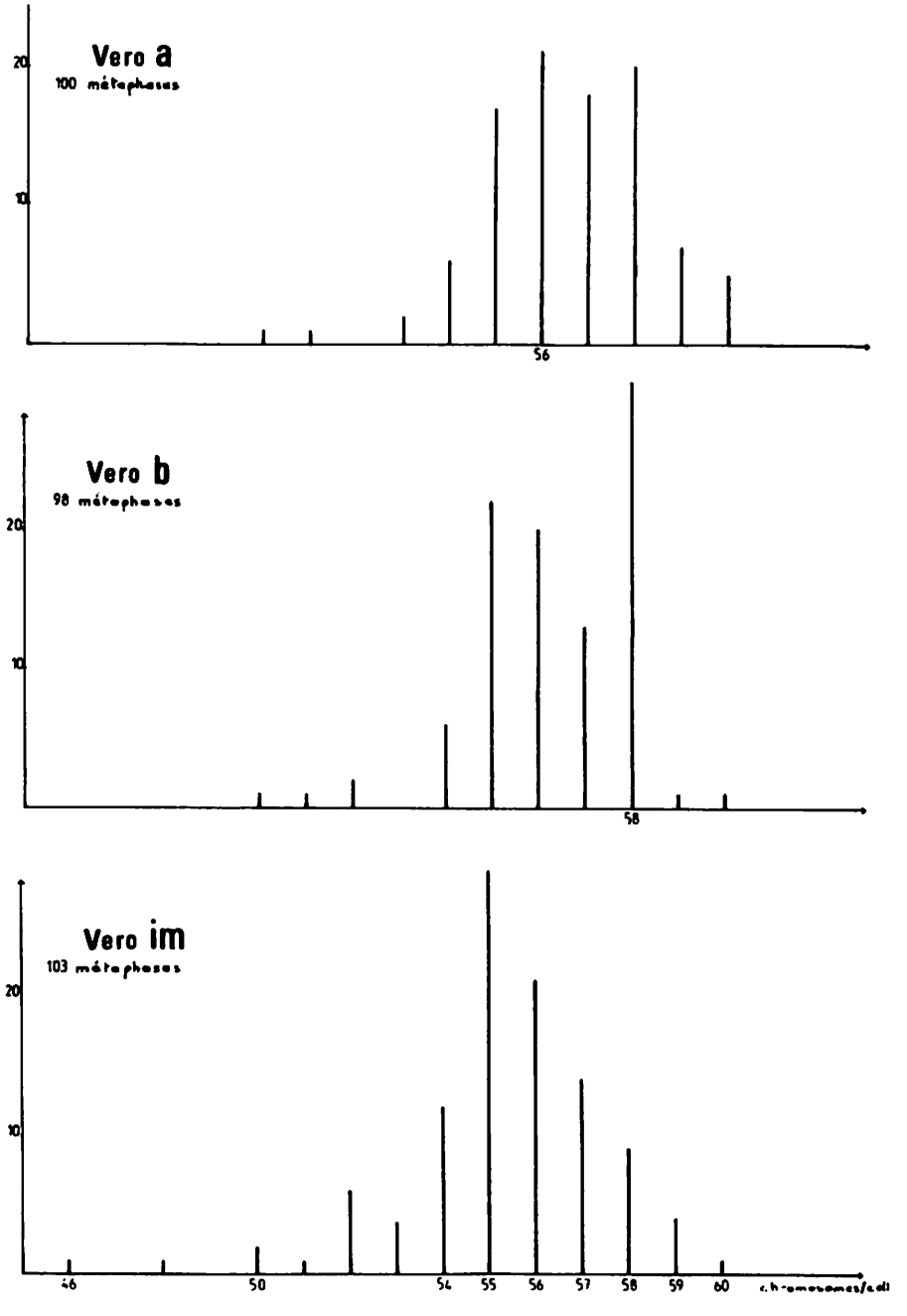

Fig. 3. Vero cells chromosomal distribution: Vero "a" is at the 131th passage, Vero "b" at the 136th passage, and Vero "I.M." at the 147th passage.

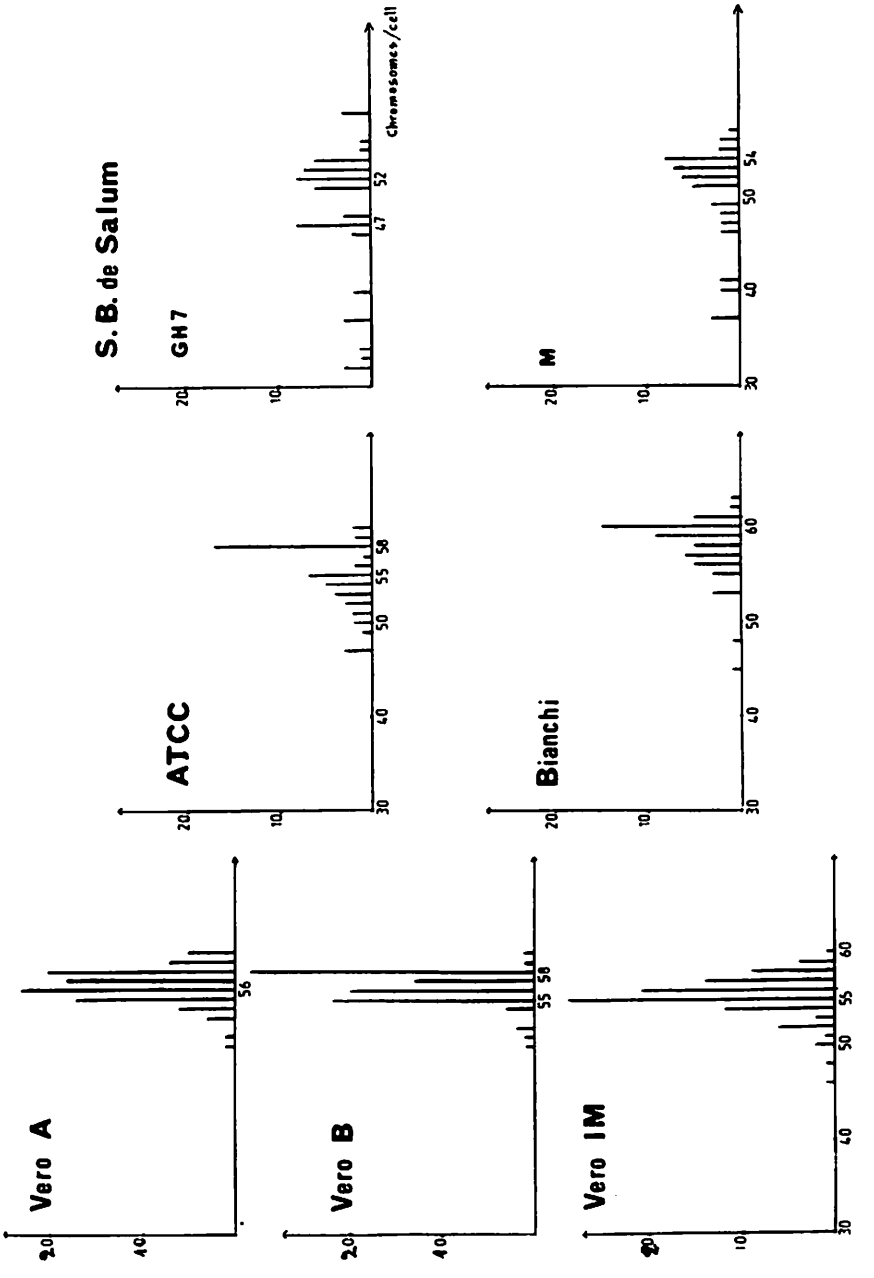

Fig. 4. Comparison of chromosomal distribution diagrams of Vero cells used at Institut Mérieux (A, B and IM) with those obtained from the literature (ATCC: American Type Culture Collection's data – Bianchi from Exp. Cell Res. 68, 253-258, 1971 – S.B. de Salum from Medicina Buenos Aires, 38, 513-518, 1978, two Vero cells lines GH 7 and M).

The other tests to verify the presence of C particles performed by Dr. Huppert's staff in Lyon were negative.

After purification we tried to detect the residual DNA in the monovalent vaccines. By molecular hybridization it was shown that the level of cellular DNA was reduced by a factor ten to the eight in several monovalents.

Finally and to conclude, we have recently obtained the French license for a killed poliovirus vaccine prepared by cultivation on Vero cell line in microcarrier system.

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Industrial-Scale Production of Inactivated Poliovirus Vaccine Prepared by Culture of Vero Cells on Microcarrier

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In 1980, the authors reported preliminary results of large-scale production of inactivated poliovirus vaccine in which virus was produced in Vero cell culture on a microcarrier. For this first stage of development, 150-liter tanks were used. The virus is now produced in 1,000-liter tanks. The main point concerning the quality of Vero cells, namely the absence of tumorigenicity, has been demonstrated, qualifying them for use in the Institut Mérieux cell bank. The purity of the cell line has also been determined by checking for the absence of bacteria, fungi, mycoplasmas, and viruses. The search for oncornavirus and for reverse transcriptase activity was carried out, and the results were negative but are not described in this paper. The quality of the purification process was checked by a search for residual cellular DNA in concentrated, purified, and inactivated vaccine. With use of a molecular hybridization procedure, a specific probe was prepared to detect ~50 pg of DNA per filter. The preliminary results show that the purification procedure fulfills the World Health Organization's requirements. T1 oligonucleotide mapping has also shown the identity of poliovirus RNA extracted from virus grown on Vero cells and that from primary monkey kidney cells. These data have led to the awarding of a license by the French government to the Institut Mérieux for production of this new, reassessed, inactivated poliovirus vaccine.

In June 1980, during the International Symposium on Reassessment of Inactivated Poliomyelitis Vaccine, at Bilthoven, The Netherlands, we presented preliminary results of large-scale cultivation of Vero cells in microcarrier culture for the production of inactivated poliovirus vaccine [1]. At that stage of our development, cells were cultivated in 150-liter tanks. Today the industrial production of Vero cell cultures and poliovirus is done in 1,000-liter tanks. We will discuss our production methods and the primary controls used to ensure the safety of Vero cells.

Production

The production flow sheet (figure 1) describes cell culture and preparation of poliovirus suspensions. Starting with 10^8 Vero cells at the 137th passage, we generally obtain 1.5×10^{12} cells in five steps at the 142nd passage.

Cells, culture media, microcarriers, and poliovirus strains presently used are identical to those described in 1981. Two slight changes have been introduced: the concentration of microcarrier is 1.5 g of Cytodex .1/liter (Pharmacia, Uppsala, Sweden), and the suspensions of working seed virus are prepared on Vero cells. For steps A, B, and C, cells are produced in glass vessels.

For step D, cells are grown in 150-liter Bilthoven units (Contact, Ridderkerk, The Netherlands). For step E, we have built our own 1,000-liter tanks, which are monitored by an automated device based on a microprocessor.

After the virus harvest is filtered through membranes with 0.2 μm pores, the virus suspension is concentrated 500-fold and purified (figure 2). The concentrated virus suspension is purified by gel filtration on Sepharose CL-6B (Pharmacia) and by ion-exchange chromatography on DEAE-Dextran-Spherosil prepared at the Institut Mérieux. After purification, the concentrated purified virus is inactivated by formalin treatment at 1:4,000, as classically done for the preparation of inactivated Salk poliovirus vaccine.

The main controversy concerning the use of Vero cells as host cells for the production of inactivated poliovirus vaccine concerns their safety. To qualify Vero cells for our working-cell bank, we tested for tumorigenicity and for the efficacy of the purification procedure in eliminating residual cellular DNA. We also have verified by T1-oligonucleotide mapping the identity on poliovirus

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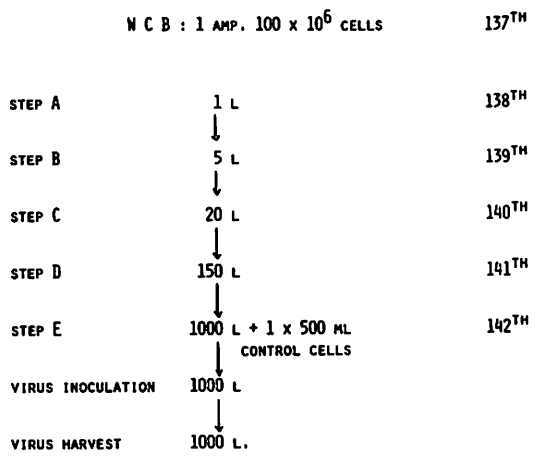

Figure 1. Flow sheet for production of inactivated poliovirus vaccine in Vero cells grown on microcarrier. WCB = working-cell bank; and amp. = ampule.

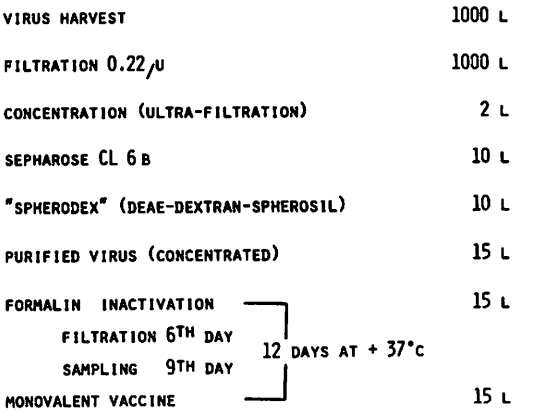

Figure 2. Purification and inactivation of poliovirus in Vero cells grown on microcarrier.

grown on primary monkey kidney cells and those grown on Vero cells.

Tests for Absence of Tumorigenicity of Vero Cells[1]

The test for tumorigenicity was described by van Steenis and van Wezel in 1982 [2]. Newborn rats at Institut Mérieux were immunodepressed with antithymocyte serum (ATS) originally prepared by van Steenis at Rijks Instituut voor Volksgezondheid (RIV), Bilthoven, The Netherlands, following the procedure of Kreeftenberg [3]. Animals were inoculated sc with 0.1 ml of ATS, in the dorsal region on days 0, 2, 7, and 14.

On day 0, each rat in groups of 10 newborn rats received 1×10^6 cells sc in 0.1 ml of inoculum in the ventral region. After 21 days, the animals were observed for presence of tumor nodules at the inoculation site. At necropsy, they were examined for presence or absence of metastasis, especially in the lungs. For all animals, samples were checked by pathologists to confirm the macroscopic examination.

Positive control animals were inoculated with Hela cells (RIV origin) in seven tests, Hep-2 cells in two tests, and KB cells in one test. Seven tests were done with Vero cells between the 138th and 147th passage. With Vero cells the results were consistently negative (table 1); neither tumor nodules nor metastasis were observed in the 70 newborn rats. On the other hand, Hela cells induced tumor nodules in the 70 animals, and pulmonary metastasis was present in 34 of 70 animals. Hep-2 cells produced tumor nodules in all 20 animals and 13 of 20 had metastasis. The results with KB cells were somewhat less positive than those for Hela or Hep-2, cells with nine of 10 animals positive for tumor nodules and three of 10 for metastasis.

The cumulative results show clearly the strong difference between Vero cells and other tumorigenic cell lines. Vero cell lines, prepared at our two working-cell banks as described and tested at lower and higher passage levels than that used for virus production, met the requirements for a nontumorigenic heteroploid cell line.

Residual Vero Cell DNA in Poliovirus Vaccine After Purification[2]

The following procedure was performed for veri-

[1] J. C. Vincent-Falquet and J. C. Moulin, Institut Mérieux.

[2] R. Martin and G. Dirheimer, Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France.

Table 1. Comparison of results of tests of tumorigenicity in newborn rats of different cell lines with Vero cells used for virus production.

Cells (no. of animals tested)	No. of animals developing	
	Subcutaneous tumoral nodules	Metastasis
Vero (70)	0	0
Hela (70)	70	34
Hep-2 (20)	20	13
KB (9)	9	3

Table 2. Results of purification on the reduction of residual cellular DNA in inactivated monovalent poliovirus vaccine grown in Vero cells on microcarrier.

Vaccine	Type	DNA/ml before purification	DNA/dose after purification	Purification coefficient
44	1	6×10^8 pg	1.031 pg	5.8×10^8
42	2	6×10^8 pg	1.143 pg	5.2×10^8
43	3	6×10^8 pg	3.692 pg	1.6×10^8

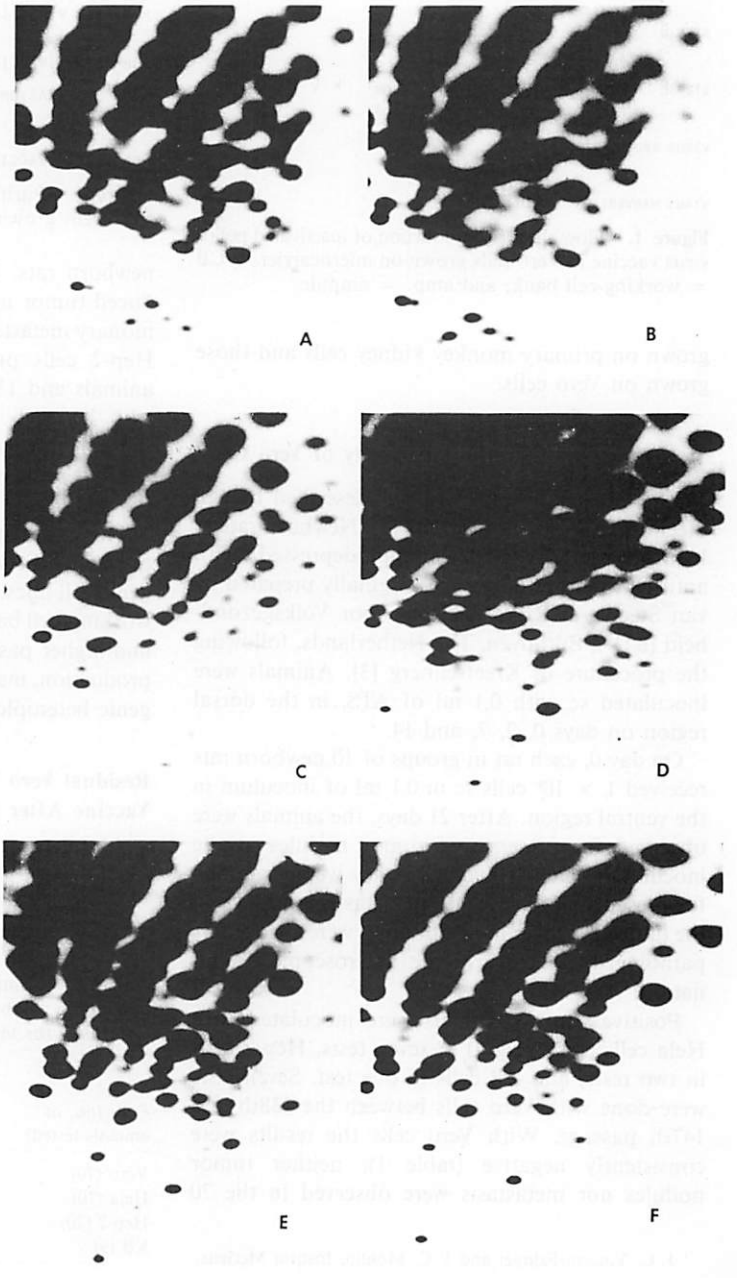

Figure 3. T1-oligonucleotide mapping of poliovirus strains (*A* and *B* = type 1; *C* and *D* = type 2; and *E* and *F* = type 3) grown on Vero cells (*A*, *C*, and *E*) and primary monkey kidney cells (*B*, *D*, and *F*).

fying the efficacy of the purification method. Nuclear and mitochondrial DNA were extracted from Vero cells. Probes were prepared by nick translation with use of four ^{32}P-radiolabeled nucleotides. For adequate sensitivity, the activity of the probes must be 2–2.8 × 10^8 cpm/μg of DNA. The vaccine samples, which were RNase treated, sonicated, denatured, and fixed on nitrocellulose filters, were then treated by molecular hybridization with the radiolabeled DNA probes.

The percentage of fixation was controlled with ^{3}H-labeled *Escherichia coli* DNA in parallel with yeast DNA, which was used as carrier. The action of formalin inactivation also was studied to verify that the range of standardization (50,000–0.05 pg of DNA per filter) was not modified by the inactivation procedure. For virologic safety, all the tests were performed on inactivated, concentrated and purified monovalent poliovirus vaccine.

The results obtained for monovalent vaccines of the three viral types clearly indicate that the reduction factor for cellular DNA is at least 10^8 from crude harvest to purification (table 2).

T1 Oligonucleotide Mapping of Types 1, 2, and 3 Poliovirus Strains Grown in Primary Monkey Kidney Cells and in Vero Cells[3]

Poliovirus strains Mahoney (type 1), MEF, (type 2), and Saukett (type 3) grown in primary monkey kidney cells (PMKC) and Vero cells, respectively, were concentrated at Institut Mérieux to a titer of at least 10^{11} $TCID_{50}$/ml. The RNA of each strain was extracted by a phenol-chloroform mixture and purified on a 15%–30% sucrose gradient in SDS buffer, as described by Lee et al. [4]. After ethanol precipitation the RNA was dissolved in 0.4 ml of sterile distilled water; 0.3 μg of RNA from each strain was digested by T1 nuclease, and 5'-terminal radiolabeling of the oligonucleotides thus obtained was carried out.

After digestion, the products were analyzed by two-dimensional electrophoresis with use of a modification of the method of de Wachter and Fiers [5]. The results are shown in figure 3. Comparison of the three paired maps shows no detectable difference between types 1, 2, and 3 produced on Vero cells and PMKC, respectively. Vero cell production of poliovirus is not associated with any detectable genetic modification.

[3] C. Bellocq, S. Van der Werf, and M. Girard, Unité de Virologie Moléculaire; Marc Girard, Institut Pasteur, Paris, France.

Conclusion

All the results of studies of the safety of Vero cells indicate a good margin of safety. The absence of their tumorigenicity in newborn rats has been demonstrated as has been the efficacy of the purification procedure and the stability of RNA poliovirus grown on Vero cells. The new inactivated poliovirus vaccine produced by culture of Vero cells in the microcarrier system is feasible on an industrial scale and fulfills the requirements of the World Health Organization [6]. Accordingly, in July 1982 Institut Mérieux was licensed by the French Government to produce poliovirus vaccine by this method.

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— List of VERO Cell-Related References —

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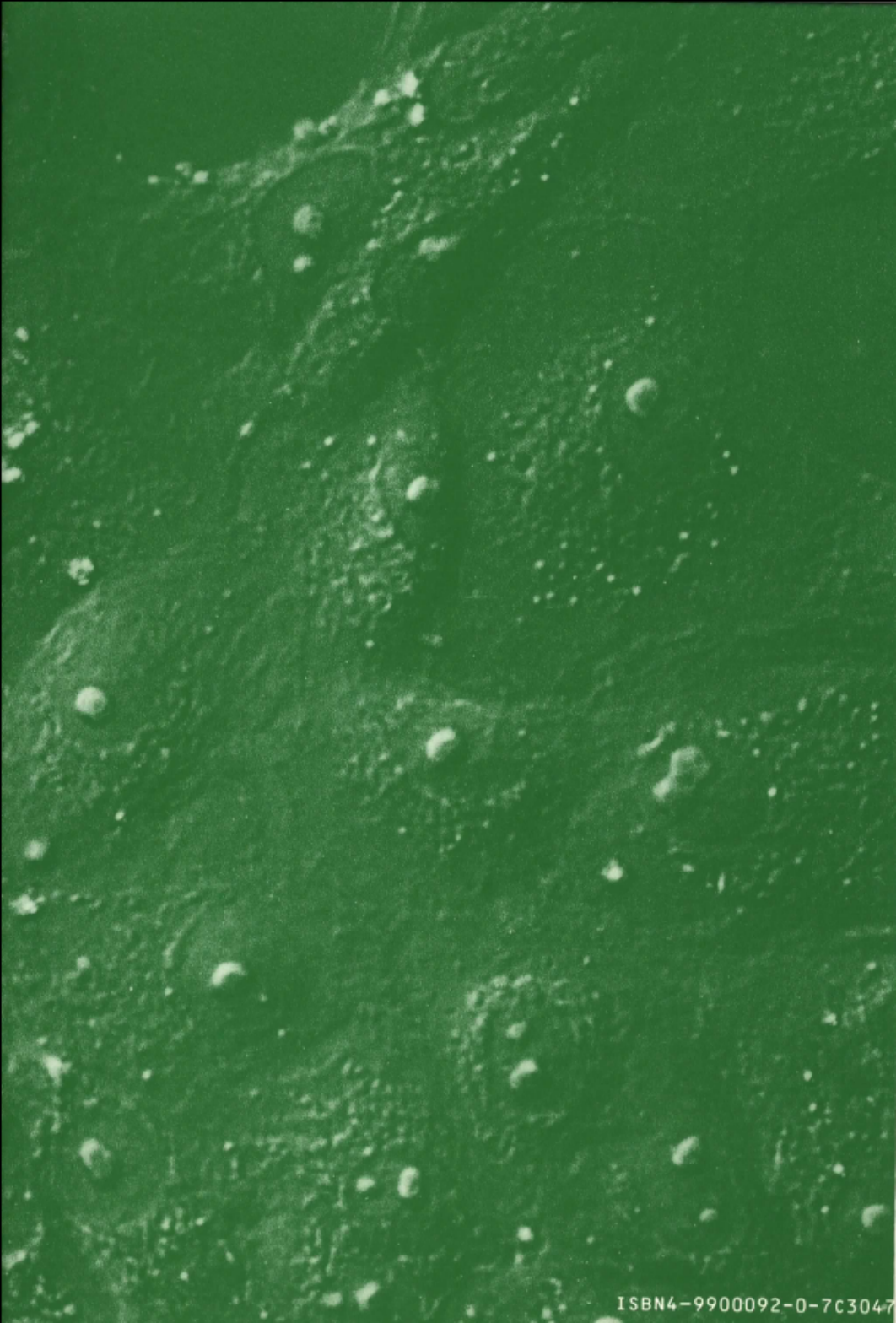

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