NEW VIRAL AGENTS RECOVERED FROM TISSUE CULTURES OF MONKEY KIDNEY CELLS

I. ORIGIN AND PROPERTIES OF CYTOPATHOGENIC AGENTS S.V.$_1$ S.V.$_3$, S.V.$_4$, S.V.$_5$, S.V.$_6$, S.V.$_{11}$, S.V.$_{12}$ and S.V.$_{15}$ [1]

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Increased use of the technique of cell cultivation for isolation, maintenance and study of viruses has resulted in the discovery of many hitherto unknown cytopathogenic agents. In screening human stool samples or rectal swabs for poliomyelitis viruses, Melnick (1) isolated several "orphan viruses" which do not type with poliovirus antiserums, and from the same source Sabin (2) obtained 5 viruses referred to as He_1, He_2, etc. The new RI series of viruses was isolated in tissue culture by Hilleman and Werner (3) from throat washings of patients with respiratory disease. Rowe, Huebner et al. (4) isolated a similar group of viruses, the APC group, which first appeared in tissue cultures of human adenoid and tonsillar tissue and later were recovered from eye washings of patients with a conjunctivitis. Recently Rustigian et al. (5) reported recovery of two cytopathogenic agents from cultures of monkey kidney cells. Thus, a number of viral agents are present in tissues and excreta of man and lower animals which defy detection by methods other than tissue culture.

During production and testing of poliomyelitis vaccine, hundreds of thousands of monkey kidney cultures prepared from thousands of monkeys were observed in our laboratories and in others participating in this program. Numerous filterable, transferable cytopathogenic agents other than poliovirus were also encountered. Although these agents were probably introduced into the cultures with the monkey kidney cells, there remains the remote possibility that some represent contaminants from human sources, horse serum, nutrient medium or other solutions used in the preparation of cultures. This report describes 8 immunologically distinct agents isolated and studied in our laboratories, and makes reference to 2 from other sources.

Materials and methods

Two types of tissue culture were used and one or more of these agents were recovered from both types. The so-called Maitland type of culture (minced tissue) was employed for large-scale production of poliovirus, whereas the new trypsinized cell type of culture introduced by Dulbecco and Vogt (6) and modified by Youngner (7) was used for all assay and safety test procedures. The latter type included both roller tube and 500-ml bottle cultures. The agents recovered from production cul-

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tures were demonstrated after poliovirus was suppressed with specific antiserum. Some of these agents were observed in roller-tube cultures beyond the end point of poliovirus titrations. The majority of isolations, however, were made in either bottle cultures or roller tubes used in safety testing of vaccine in which a large number of normal cultures (i.e., those on tests with vaccine) were held, examined and subcultured over a period of 21 days. After these agents were isolated, subcultured several times, and titrated to remove the possibility that merely nonspecific toxic factors were transferred, their antiserums were prepared as follows: emulsified antigens were prepared with living viruses in mineral oil according to the procedure described by Salk and Laurent (8) in which Arlacel A is used as the emulsifying agent. Albino rats, weighing 250 grams, were given one injection of 0.5 ml of the emulsified antigen in each hind leg or 2-to-3-pound white rabbits were given 2.5 ml, or both were done. The animals were bled by cardiac puncture after a 3- to 4-week interval. In most cases animals were not exsanguinated at the first bleeding, but remained alive as a reservoir for future antiserum needs. In some instances, booster doses of aqueous antigen were given after 6 months or longer. Antiserums so prepared were titrated in monkey kidney cell cultures against 100 $TCID_{50}$ (median tissue culture infective dose) of the homologous virus. Serums which at dilutions of 1 in 64 or greater protected cells were considered adequate for routine typing of other isolates.

The agents isolated will be referred to as "Simian viruses" (S.V.) until such time as a definite association with some other host or identification can be established. These agents will be referred to as $S.V._1$, $S.V._2$, etc. Of the original group of agents which included $S.V._1$ through $S.V._{15}$, eight ($S.V._{1, 2, 4, 5, 6, 11, 12}$ and $_{15}$) will be described in this paper. The missing numbers were held by agents that were identified or reclassified after antiserum studies, or which have not yet been adequately studied for inclusion at this time. The latter category includes $S.V._{13}$ which is the "lacy or foaming" agent frequently seen in cultures of monkey kidney cells.

Results

The following is a brief account of the original isolation and description of the 8 agents referred to above.

$S.V._1$ was recovered from roller-tube cultures of trypsinized monkey kidney cells planted on February 8, 1954. This series included 1,700 tubes prepared from a pool of kidneys from 4 apparently healthy cynomolgus monkeys. After 6 to 8 days of incubation the cultures appeared satisfactory and were used for titrations of poliovirus. After 6 more days of incubation a type of cell destruction was seen in some cultures beyond the end point of the poliovirus that was atypical of the damage produced by the latter. This atypical damage was observed in 17 per cent of the cultures. This cytopathogenic effect (C.P.E.) was characterized by a reduction in size and distinct rounding of the cells. Infected cells were generally seen in clusters; they remained affixed to the glass rather than floating free as do cells infected and destroyed by polioviruses. The $S.V._1$-infected cells were also characterized by very discrete black borders. It was possible to transfer this specific C.P.E., which had a titer of $10^{-8.5}$ $TCID_{50}$, to other kidney cultures. After several tissue culture

passages, the extent of damage mounted to complete destruction of the culture and the titer also increased. S.V.$_1$, currently in its twentieth passage, has a titer of 10^{-6} $TCID_{50}$ or greater following 7 to 10 days of incubation in trypsinized monkey kidney cultures. S.V.$_1$ was studied in greater detail than others isolated more recently. Therefore, a larger volume of information concerning S.V.$_1$ is on hand and will be published later.

S.V.$_2$, like S.V.$_1$, was first recovered from roller-tube cultures used for assay of poliovirus and was again observed in cultures beyond the end point of the poliovirus. This agent was obtained from cultures of rhesus monkey kidney cells planted on August 31, 1954. Unlike S.V.$_1$, it did not require a period of adaptation to tissue culture; it completely destroyed cultures in 1 day when subcultured undiluted. After 3 such passages it titered at $10^{-6.3}$ $TCID_{50}$. The characteristic C.P.E. of S.V.$_2$ was seen only in very early stages of cell infection when relatively few small patches of cells were involved. These infected cells had very discrete borders, were somewhat pear-shaped or resembled budding yeast cells, and were smaller than normal cells. After more extensive degeneration had occurred, the specific or characterizing C.P.E. was no longer evident; however, the damage was still unlike that produced by poliovirus. Since its original isolation S.V.$_2$ has been recovered occasionally from production cultures and from other assay cultures. It has been associated only with cultures of rhesus monkey kidney cells.

S.V.$_4$ was first observed in bottle cultures used in vaccine safety tests that had been planted with rhesus monkey kidney cells on December 7, 1954. This agent, like S.V.$_2$, required little, if any, adaptation to growth in tissue culture since in the first passage dilutions of 10^{-1} completely destroyed the cultures within 3 to 6 days. After 3 successive tissue-culture passages, it had a titer of 10^{-6} $TCID_{50}$ or greater. The most characteristic C.P.E. of S.V.$_4$ was seen in more advanced stages of cell destruction. The cells became very granular, contained a few small vacuoles, and the nucleus was obliterated. The cells did not become round, nor was the cell wall or border accentuated as described in S.V.$_1$ and S.V.$_2$ infections. The granulation and vacuolization gave cells a rather riddled, or feather-like, appearance. Destroyed cells did not slough off the glass quite as readily as did cells destroyed by poliovirus, and often they could be seen still fastened by one cell process. This condition tended to increase the feather-like appearance because of a waving or fluttering motion of the cells as the medium was agitated during microscopic observation. In early stages of infection with S.V.$_4$ small patches of infected cells generally were seen at the periphery of the cell sheet in roller-tube cultures. These cells were enlarged or swollen and the first evidence of granulation and vacuolization described above was seen in some cells. In addition to possessing the specific C.P.E., S.V.$_4$ was characterized by its inhibition by poliovirus antiserums and by normal serums from several animal species even when high titered virus was used.

S.V.$_5$ was recovered in roller-tube cultures of trypsinized rhesus monkey kidney cells which had been inoculated with fluid from a vaccine safety test bottle culture prepared on October 8, 1954, with kidney cells from rhesus monkeys. The C.P.E. produced by S.V.$_5$ was quite

unlike that described for the preceding agents, and even after 7 successive passages there was not complete degeneration of the cultures. In the early stage of cell infection with this agent there were patches of fused or coalesced cells which contained several nuclei and resembled giant cells. This observation was not unlike that seen in cultures infected with measles (9) or mumps (10) viruses, but the vacuolated or lacy appearance of the cells in the latter two infections did not follow. These enlarged cells, or clusters of cells, became quite granular; their nuclei were lost or obliterated, and they floated free in the medium, appearing only as somewhat transparent amorphic masses of degenerated protoplasm. Other, uninfected, cells remained affixed to the glass and appeared healthy. Titers of $10^{-6.5}$ $TCID_{50}$ or greater have been obtained in monkey kidney cultures. After 7 tissue-culture passages, $S.V._5$ was placed in storage at -78 C, where it was apparently destroyed as attempts to infect cultures with thawed material at later dates failed. Recently, however, $S.V._5$ was reisolated and identified with antiserums prepared from the original isolate.

The origin of $S.V._6$ was somewhat vague. This agent first occurred in our tissue-culture research laboratories in cultures of rhesus monkey kidney cells planted on January 11, 1955, which were employed in a poliovirus neutralization test on a human postvaccination serum. The C.P.E., unlike that produced by poliovirus, was seen in cultures challenged with type 1 Mahoney virus, but which were protected from poliovirus by type 1 antibodies in the serum. It was not observed in cultures challenged with type 2 MEF or type 3 Saukett viruses. Further studies on the patient's serum alone failed to show evidence of virus in it or of antibodies against this agent. When the Mahoney standard virus, prepared on July 10, 1954, was re-examined with specific antiserums to inhibit poliovirus, this agent was again recovered; thus it apparently had been introduced into the neutralization test along with the challenge virus. This standard virus, however, had been in use for about 6 months and had not previously shown evidence of this contaminant. When it was first observed, the C.P.E. of $S.V._6$ was not like that produced by poliovirus but somewhat resembled that seen in cultures infected with herpes virus. The latter produces a large rounded cell with a prominent nucleus and with several spike-like processes.

Since herpes virus was being studied at that time, $S.V._6$ was immediately typed with herpes antiserum, but could not be neutralized with it. In addition, 10 mice were inoculated intracerebrally with this agent, and 10 mice with herpes virus for controls. All animals in control group succumbed to herpes encephalitis, whereas the 10 mice given $S.V._6$ remained healthy. After additional tissue-culture passages the C.P.E. of $S.V._6$ ceased to resemble that of herpes virus and become more like that caused by poliovirus in its early stages. The agent was further characterized by the fact that it seldom destroys more than 25 to 30 per cent of the cells in the culture. These infected cells slough off the glass, disintegrate, and the remainder of normal cells repopulate the culture, so that in some cases after 7 days one might not recognize that change had occurred in the culture.

$S.V._{11}$ was originally isolated from safety test bottle cultures prepared on March 2, 1955. The type of C.P.E.

produced by S.V.$_{11}$ is indistinguishable from that caused by S.V.$_1$. S.V.$_{11}$, however, was recovered from rhesus monkey kidney cells, whereas S.V.$_1$ was isolated in cultures prepared from cynomolgus monkey kidneys. S.V.$_{11}$ did not require a period of adaptation to growth in tissue culture as did S.V.$_1$, and it had a higher titer. After 9 passages its titer was $10^{-7.5}$ $TCID_{50}$. In neutralization tests there was no cross-over between these two agents. It is interesting that this agent had not been encountered prior to the middle of March, 1955, but was reisolated numerous times during the spring of 1955.

S.V.$_{12}$ was recovered from safety test bottle cultures prepared on April 20, 1955, but it probably occurred earlier and was confused with S.V.$_4$. This agent had a C.P.E. identical with that produced by S.V.$_4$, but unlike S.V.$_4$, it was not inhibited by normal serums. Titrations of a pool of S.V.$_4$ which was inhibited by normal serums, and of S.V.$_{12}$, which was not inhibited, revealed the S.V.$_4$ pool to have the higher titer, thus demonstrating that inhibition by serum was not a function of the virus titer. In a cross-neutralization test between S.V.$_{12}$ and S.V.$_4$ and their serums, no evidence was obtained that S.V.$_{12}$ was immunologically similar to S.V.$_4$.

S.V.$_{15}$ was isolated in the middle of March, 1955, in cultures of rhesus monkey kidney cells. The type of cell damage produced by S.V.$_{15}$ was essentially indistinguishable from S.V.$_1$ and S.V.$_{11}$ in that it was typified by rounding of cells with cluster formations which did not readily slough off the glass wall of the tube. It did produce cell damage in a shorter period of time than did S.V.$_1$ and reached higher titers than either S.V.$_1$ or S.V.$_{11}$. Average titers have varied between $10^{-7.5}$ and $10^{-8.5}$ $TCID_{50}$. In cross-neutralization tests it was shown to be immunologically distinct from S.V.$_1$ and S.V.$_{11}$.

The characteristic C.P.E. produced by these agents has been used very effectively in their identifications. Some can be identified quite regularly by this method while others can be placed in certain groups. For convenience these groups are as follows:

Group I	Group II	Group III	Miscellaneous
S.V.$_1$	S.V.$_2$	S.V.$_4$	S.V.$_5$
S.V.$_{11}$	S.V.$_{15}$*	S.V.$_{12}$	S.V.$_6$
S.V.$_{15}$		S.V.$_{59}$*	

* See table 4.

During preparation of this paper at least two more viruses, apparently different from those described here, have been isolated. These are being studied further.

In addition to the observations on these viruses in trypsinized monkey kidney cell cultures, they were studied for growth in monkey testicular cells, human kidney cells, HeLa cells and in Maitland type cultures of monkey kidney tissue. They were also filtered through 03 Selas filters under pressure and tested for heat inactivation at 65 C for 30 minutes. The time required to reduce titers to less than 1 $TCID_{50}$ by 1 in 4,000 formaldehyde at 37 C was determined. To test the samples, formaldehyde was neutralized with sodium bisulfite and the complex removed by dialysis. The results of these experiments are summarized in table 1.

With the exception of S.V.$_2$, very little, if any, cross-neutralization was seen among these 8 monkey viruses and the 3 types of poliovirus. The data from cross-neutralization tests are given in table 2. S.V.$_2$ appeared to have some antigenic similarity to S.V.$_1$, S.V.$_4$, and to S.V.$_5$; however, this was of low order.

TABLE 1

General properties of Simian viruses

Viruses	Growth in cultures of				Inactivated by 65 C, 30 min.	Time (*hours*) required to reduce titer to 1 ID_{50} by HCHO	O_2 Seitz filterable	Trypsin cell culture (av. titer)
	Monkey testis	HeLa	Human kidney	Maitland				
S.V.$_1$	Yes	Yes*	Yes	Yes	Yes	<48	Yes	$10^{-6.0}$
S.V.$_2$	No	No	No	Yes	No	<48	Yes	$10^{-6.3}$
S.V.$_4$	Yes*	Yes*	No	Yes	Yes	<48	Yes	$10^{-6.0}$
S.V.$_5$	No	No	No	Yes	Yes	<24	Yes	$10^{-6.5}$
S.V.$_6$	No	No	No	Yes	No	<48	Yes	$10^{-6.5}$
S.V.$_{11}$	Yes	Yes	Yes	Yes	Yes	<24	Yes	$10^{-7.5}$
S.V.$_{12}$	Yes	Yes	Yes	Yes	No	<96	Yes	$10^{-6.0}$
S.V.$_{15}$	Yes	Yes	Yes	Yes	Yes	<48	Yes	$10^{-7.5}$
Type 1 Mahoney	Yes	Yes	Yes	Yes	No	<96	Yes	$10^{-7.5}$

* Cultures destroyed in first passage only.

When tested against human gamma globulin, only S.V.$_{11}$ was neutralized. The gamma globulin inhibited the virus in dilutions up to 1 in 8. Naturally occurring antibody was demonstrated in several individual monkey serums against some of these viruses. Each virus was also typed with B virus, with type 1 and type 2 dengue fever virus antiserums, and with Sabin's human enteric virus antiserums, all very kindly made available to us by Dr. Albert B. Sabin. Neutralization of S.V.$_1$ was observed in type 2 dengue antiserum. This possible identification of S.V.$_1$ as type 2 dengue virus was eliminated when preimmunization serum from the cynomolgus monkey used to produce the type 2 dengue antiserum showed the same anti-S.V.$_1$ titer as did the post-dengue immunization serum from the same monkey.

TABLE 2

Cross neutralization tests

Serums	Viruses, 100 $TCID_{50}$										
	S.V.$_1$	S.V.$_2$	S.V.$_4$	S.V.$_5$	S.V.$_6$	S.V.$_{11}$	S.V.$_{12}$	S.V.$_{15}$	Mah.	MEF_1	Sauk.
S.V.$_1$	1:1,024	1:8	0	0	0	0	0	0	0	0	0
S.V.$_2$	0	>1:256	1:4	0	0	0	0	0	0	0	0
S.V.$_4$	0	1:4	>1:256	0	0	0	0	0	0	0	0
S.V.$_5$	0	1:8	0	1:64	0	0	0	0	0	0	0
S.V.$_6$	0	0	0	0	1:64	0	0	0	0	0	0
S.V.$_{11}$	0	1:4	1:4	0	1:4	1:256	0	0	0	0	0
S.V.$_{12}$	0	0	0	0	0	0	1:32	0	0	0	0
S.V.$_{15}$	0	0	0	0	0	0	0	1:32	0	0	0
Mahoney	0	0	0	0	0	0	0	0	1:128	0	0
MEF_1	0	0	0	0	0	0	0	0	0	1:256	0
Saukett	0	0	0	0	0	0	0	0	0	0	1:128

When tested against human enteric virus rabbit antiserums, S.V.$_4$ was inhibited by anti-He$_2$ and He$_5$, but not by He$_{1, 3}$ and $_4$. S.V.$_{12}$ was inhibited by anti-He$_5$. Since S.V.$_4$ was frequently inhibited by normal serums and heterologous antiserums, the significance of its neutralization by serum prepared against He$_2$ and $_5$ viruses was doubtful. Inhibition of S.V.$_{12}$ by anti-He$_5$ at first seemed to be significant. However, when pre-He$_5$ immunization serum from the same animal was tested it also inhibited S.V.$_{12}$. Thus a rabbit appeared to have natural antibody against this agent.

All of these monkey viruses in the culture medium fixed complement by the Bengtson (11) method in the presence of specific antiserum. In hemagglutination studies by the Salk (12) pattern method, S.V.$_5$ and S.V.$_{15}$ produced hemagglutination of chicken erythrocytes at room temperature. This hemagglutination was also inhibited by specific antiserums. S.V.$_{2, 4, 12}$ and $_{15}$ all hemagglutinated rhesus and cynomolgus monkey erythrocytes at 5 C, whereas only S.V.$_{12}$ and S.V.$_{15}$ hemagglutinated human type 0 erythrocytes.

Monkeys were inoculated intracerebrally, intramuscularly, and in some instances by other routes, with all the viruses. Serum from each monkey inoculated was first assayed for antibodies against the specific virus to be injected. Only negative animals were used. With the exception of S.V.$_{12}$ and S.V.$_{15}$ no evidence of clinical illness or of gross or histopathological lesions was seen. Monkeys inoculated intracerebrally with high titer undiluted S.V.$_{12}$ and S.V.$_{15}$ viruses succumbed within 4 to 6 days after inoculation. The specific type of virus inoculated was readily recovered from brain and cord tissues of these animals. These same two viruses, however, when inoculated intramuscularly into monkeys failed to produce clinical disease or gross or histopathological lesions. The histopathological lesions observed in the intracerebrally inoculated monkeys revealed necrosis and complete destruction of the choroid plexus plus a generalized aseptic type of meningitis. No other brain pathology or lesions resembling those of poliomyelitis were seen. The various organs studied exhibited no recognized lesions and it appeared that all involvement was limited to the central nervous system in the region of the virus inoculation. The lesions produced by S.V.$_{12}$ and S.V.$_{15}$ were indistinguishable.

Although no recognizable disease was produced in the monkeys inoculated with these viruses, with the above noted exceptions, some evidence was obtained in our laboratories, and in others, that S.V.$_2$ and S.V.$_6$ may have some etiological significance in enteric infections. In table 3, showing the serological identification of virus samples received from other laboratories, it is noted that sample M.B.$_3$ from the Army Medical Service Graduate School (A.M.S.G.S.) was identified as S.V.$_2$. Agent M.B $_3$ was isolated by Dr. Viola M. Young (Department of Bacteriology, A.M.S.G.S.) from stool specimens from monkeys at the Okatie Farm monkey colony during an epidemic of diarrhea. Recently in our laboratory a small (approximately 3 pounds) cynomolgus monkey, which had been in the laboratory for at least 6 months, became ill with diarrhea and died. A culture from a rectal swab taken the day before death revealed the presence of S.V.$_6$ in high concentration. Additional infectivity studies with S.V.$_2$ and S.V.$_6$ given monkeys by the oral route are in progress.

TABLE 3

Serological identification of samples from other laboratories

Submitted by	Total no of samples	No. identified in column 4	Identification
Connaught Labs.	7	5	S.V.$_4$
		2	S.V.$_{11}$
Parke Davis Labs.	22	4	S.V.$_{11}$
		16	S.V.$_{12}$
		2	B virus
Cutter Labs.	3	1	S.V.$_4$
		1	S.V.$_{11}$
		1	S.V.$_6$ + type 2 polio + S.V.$_{16}$
Pitman Moore Labs.	3	2	B Virus
		1	Type 1 + 2 polio
Biologic Control, N.I.H.	7	6	No definite agent demonstrated
		1	S.V.$_5$?
Rustigian, Univ. of Chicago	2	0	Unidentified
Army Med. Serv. Grad School	3	1	S.V.$_2$
		1	S.V.$_5$
		1	Unidentified, named S.V.$_{59}$
Sharp and Dohme Labs.	1	0	Unidentified, named S.V.$_{16}$

Sample no. 59 in table 3, also from the Army Medical Service Graduate School, produced a C.P.E. very similar to that produced by S.V.$_{12}$ but was not identical serologically. This no. 59 virus was isolated by Miss Nancy G. Rogers (Department of Virus and Rickettsial Diseases, A.M.S.G.S.) from the lung of a monkey with a respiratory illness. Although agent no. 59 could not be identified as one of the S.V. agents isolated in our laboratories, it undoubtedly belongs in this group; therefore, with the consent of Miss Rogers, it has been given the identification of S.V.$_{59}$.

A virus sample received from the Sharp and Dohme Laboratories produced a C.P.E. almost identical to that of S.V.$_2$ but was neutralized by S.V.$_2$ antiserum in a dilution of 1 in 4 only. Therefore, it was similar to, but not identical with, S.V.$_2$. This agent was isolated in cultures of rhesus monkey kidney cells by Miss Evelyn Dwyer and with her consent has been given the identification of S.V.$_{16}$. No further information is available at this time.

None of these monkey viruses infected embryonated eggs as determined by death of the embryo. However, one agent from the A.M.S.G.S. identified as S.V.$_5$ had been recovered from embryonated eggs inoculated with monkey kidney tissue-culture fluids. Groups of adult and suckling mice were inoculated intracerebrally and intraperitoneally with each virus. However, no evidence of disease nor death of the mice was observed. Rats and rabbits which were inoculated with live virus for the production of antiserum also did not succumb to infection with any of these agents.

A complete record of the relative frequency of the isolation of these 8 viruses in all sections of our laboratories is not readily available. However, table 4 shows the degree with which each agent was encountered during vaccine safety testing from January, 1954, through January, 1956. The column headed "number of isolations" refers to the number of safety tests in which one or more isolations of a given virus was made and does not include multiple recoveries in any one test. The degree of contamination with these viruses

TABLE 4

Frequency of contamination during 424 safety tests

Viruses	No. of safety tests contaminated
S.V.$_1$	0
S.V.$_2$	5
S.V.$_4$	72
S.V.$_5$	22
S.V.$_6$	0
S.V.$_{11}$	11
S.V.$_{12}$	37
S.V.$_{15}$	9
Not identified*	30

* None of the 30 isolations listed as unidentified were suspected of being agents other than one or another of this group of 8 S.V. agents. They were not, however, differentiated by neutralization because of problems of time aand feility.

varied from one or two cultures to over one half of those employed in a given test. It is seen in table 4 that S.V.$_4$ was our most frequent isolate. There did appear to be a seasonal, or simulated seasonal, variation involved in the appearance of these agents, as most of the S.V.$_4$ isolations were made between January and the middle of April. It did not appear again until January, 1956. From the middle of April to August 1, S.V.$_{11}$ occurred with the greatest frequency, although it was not previously seen at all. S.V.$_{12}$ and S.V.$_{15}$ first appeared about the same time as S.V.$_{11}$ and have been encountered occasionally during the same time period. S.V.$_5$ first appeared in early October, 1954, and was isolated several times during that fall but has been reisolated only once until September and October, 1955, in which two months 19 isolations were made.

Other laboratories producing and testing poliomyelitis vaccine and working on some phase of the program have encountered similar viruses, and have very kindly made available to us samples of these agents for serological comparison with the 8 viruses described in this paper. Table 3 summarizes the results of the serological typing of these agents against our 8 monkey virus antiserums as well as against other antiserums where typing was done in our laboratory to confirm the identification of known viruses or of mixtures which had caused difficulty in identification.

It can readily be seen in table 3 that all samples tested could be identified as one or the other of our "simian viruses" or other known viruses, with the exception of those received from Rustigian, from the National Institutes of Health, one from the Army Medical Service Graduate School and one from Sharp and Dohme. More recent monkey virus isolations, not included in this paper but referred to previously, probably contain an agent similar to those previously described by Rustigian. These agents exhibited a low order of pathogenicity for monkey kidney cells in tissue culture, and did not make their presence as obvious as did the 8 agents described here.

In addition to these apparently new monkey viruses, B virus was isolated in tissue culture on one occasion from the brain of a rabbit inoculated with virus fluid from the first stages of production. It was also of interest that one series of roller-tube cultures planted with kidney cells from cynomolgus monkeys contained a low order of contamination with a trichomonad. The species of this protozoan was never determined.

DISCUSSION

The isolations and characteristics of 8 apparently new viruses have been described. Although definite proof has

not been presented, these agents probably have been simian in origin. What diseases, if any, they might be responsible for in monkeys also has not been established. S.V.$_2$ and S.V.$_6$ may be etiological agents in intestinal infections of monkeys inasmuch as both have been isolated from stool samples of animals suffering from diarrhea. None have produced experimental infections when inoculated intramuscularly although both S.V.$_{12}$ and S.V.$_{15}$ caused a prostrating type of paralysis and death in monkeys following intracerebral inoculation. Attempts which are being made to infect monkeys by other routes of inoculation are not yet completed.

There has been no evidence to indicate that any of these 8 viruses might be of public health concern, although S.V.$_{11}$ was neutralized by human gamma globulin, S.V.$_{12}$ and S.V.$_{15}$ hemagglutinated human erythrocytes, and S.V.$_1$ S.V.$_{11}$, S.V.$_{12}$ and S.V.$_{15}$ infected and destroyed human kidney and HeLa cells in tissue culture. Sufficient serological study has not been done to differentiate these agents from all known human pathogens occurring in the United States, but the characteristics of the growth of these viruses (with the exception of S.V.$_5$) in tissue culture and their failure to infect mice, rats, rabbits and embryonated eggs have served to rule out most possibilities. In addition it has not been possible to identify any of these agents as dengue fever virus, a virus common to the areas in which most of the monkeys have been trapped, nor have any of the 8 been shown to be B virus.

The greatest significance of these viruses has been their appearance in tissue cultures used to produce and to test poliomyelitis vaccine. This has been especially troublesome during tissue-culture safety testing of the vaccine as the occurrence of these viruses has in many instances invalidated tests for poliovirus, making retesting necessary and thereby delaying the release of vaccine. There has also been the problem of proving in some cases that the contaminating "simian virus" isolated during a vaccine safety test was not in the vaccine sample under test. In general, however, this has not been a problem since many of these agents have been recovered from normal or uninoculated control cultures. The likelihood of the contaminating S.V. agent being in the vaccine sample has also in many instances been diminished by a statistical consideration of the results of all the tests given a particular lot of vaccine. Five tissue-culture safety tests have been done on each lot of vaccine with 800 to 1,800 ml of vaccine being tested in each of the 5 tests. In nearly every instance different cell preparations have been used in the cultures employed in each of the 5 tests. Thus, if, for example, S.V.$_4$ had been recovered in a large number of cultures in one test but did not occur in any of the other tests, it seemed unlikely that it could have been in the vaccine under test. Perhaps the most convincing argument against the possibility of there being live "simian viruses" in the vaccine was obtained through the studies on formaldehyde inactivation of these agents. As was shown in table 1, all but S.V.$_{12}$ were reduced to less than 1 $TCID_{50}$ in less time than was required for poliovirus, and S.V.$_{12}$ was inactivated in the same time period as poliovirus. Therefore, if it was shown by the safety test that the high titered poliovirus had been inactivated by the formaldehyde treatment then certainly any trace amounts of a "simian virus"

possibly present in the production pool of poliovirus would also have been inactivated.

The data on attempts to grow these viruses in the Maitland type culture, as seen in table 1, indicated that all 8 viruses could be cultivated in this type of culture when a large inoculum was employed. In the trypsinized cell cultures where most of these agents have been recovered the presence of the contaminating virus was generally not detected until the cultures were at least 14 days old from the time of planting. Since the Maitland cultures used in production of poliovirus were never held more than 10 days and were undergoing degeneration by poliovirus during the last 4 days of their incubation it seemed unlikely that the trace amounts of "simian viruses" carried into the cultures with the kidney tissue (or blood) could have multiplied to high titers. A large number of production samples has not been studied for S.V. contamination but of those that have been assayed a few have been found to contain $S.V._2$ only. Table 4 indicates that $S.V._2$ was recovered only 5 times in the 186 tests contaminated with these agents and this again casts doubt on the possibility of the "simian viruses" being in the vaccine under test.

It should be mentioned that in addition to the problems presented by the occurrence of these viruses in tissue-culture safety tests, they can also be a source of difficulty when the pH or metabolic inhibition test is employed. The metabolic inhibition test in tissue culture demonstrates only decreased metabolism of the cells without giving the observer an indication of the cause of inhibition On the other hand, when these viruses have occurred in poliovirus neutralization tests done in roller-tube cultures, they were recognized by their characteristic C.P.E.

It was felt that the experimental evidence and conclusions presented here left very little concern over the possible presence of live "simian viruses" in the Salk type poliomyelitis vaccine currently in production and being used for immunization of children.

Summary

Eight apparently new submicroscopic, filterable, cytopathogenic agents recovered in tissue cultures of rhesus and cynomolgus monkey kidney tissue have been described. No definite associations have been made between any of these agents and naturally occurring diseases of monkeys or other animals. The significance of these viruses in the poliomyelitis vaccine program and the problems created by them during vaccine safety testing in tissue culture have been discussed.

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