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CHARACTERIZATION OF SIMIAN VIRUSES ISOLATED FROM WILD CYNOMOLGUS MONKEYS

M. SUZUKI, *F. NISHIKAWA, A. SASAGAWA, T. INAYOSHI

Tsukuba Primate Center for Medical Science, National Institute of Health, Hachimandai, Yatabe-machi, Tsukuba-gun, Ibaraki-ken, 305, and *Department of Virology and Rickettsiology, National Institute of Health, Kamiosaki, 2-chome, Shinagawa-ku, Tokyo 141, Japan

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Summary. — Twenty three strains of simian viruses isolated from nasopharyngeal swabs and respiratory tissues of cynomolgus monkeys showing mild symptoms of respiratory disorders were classified into three groups by their biological, serological and morphological characteristics. Thirteen strains classified as group 1 were judged to be enteroviruses. Four strains of group 2 and six strains of group 3 were identified as simian adenoviruses. Yields of the ten simian adenoviruses of groups 2 and 3 were compared in three continuous cell lines after several serial passages.

Key words: simian viruses; cynomolgus monkey; virus isolation and identification; biological, serological and morphological properties

Introduction

Several investigators have reported that many simian viruses were isolated from respiratory and intestinal tracts or other tissues of nonhuman primates (Hoffert *et al.*, 1958; Tyrrell *et al.*, 1960; Hsiung *et al.*, 1966; Kim *et al.*, 1967; Hull, 1968; Eugster *et al.*, 1969; Hillis *et al.*, 1969; Soike *et al.*, 1971; Basnight *et al.*, 1971; Heberling, 1972*a*, *b*; Kalter *et al.*, 1971, 1973, 1982; Hooks *et al.*, 1975; Vasilleva *et al.*, 1978; Shroyer *et al.*, 1979; Darai *et al.*, 1980). Previously, we followed the epidemiology of myxo-paramyxovirus infections among wild cynomolgus monkeys imported into the National Institute of Health from Southeast Asian countries through February to August, 1973 (Nishikawa *et al.*, 1974). In that study, we have isolated parainfluenza type 3 virus, SV5 and more than twenty strains of unidentified viruses from nasal and throat swabs and from some organs of monkeys suffering from mild respiratory disorders or dying of similar symptoms. Later on, out of those unidentified isolates, four were identified as Murayama virus, a newly discovered paramyxovirus (Nishikawa *et al.*, 1977). In this study, we attempted to group the rest of unidentified viruses according to their biological, serological and morphological properties and to examine the growth spectra of several selected strains in three kinds of cell lines.

Materials and Methods

Virus. In the previous work, the viruses were isolated in primary cynomolgus monkey kidney (MK), HEp-2 cell cultures and in embryonated eggs (Nishikawa *et al.*, 1974). From these, the viruses isolated in MK and HEp-2 cells were dealt with in the present study.

Cells and media. MK cells were grown in Earle's balanced salt solution supplemented with 0.5% lactalbumin hydrolysate (LE), 2% calf serum and maintained in LE supplemented with 0.1% yeast extract. HEp-2 cells were grown in Eagle's minimum essential medium (MEM) containing 5% calf serum and maintained in Eagle's MEM. JINET (Tsuchiya *et al.*, 1963), Vero and R-66 (Taguchi *et al.*, 1979) cells were grown in Eagle's MEM supplemented with 10% foetal calf serum and maintained in the same medium supplemented with 2% bovine plasma albumin.

Antisera. Antisera were prepared in rabbits inoculated with each supernatant of frozen and thawed MK cell suspension infected with the virus. The rabbits were given 5 ml of the materials intravenously twice a week. The inoculation was repeated eight to ten times. The rabbits were bled one week after the last inoculation.

Neutralization test. Neutralization tests (NT) were performed in MK cell cultures, that is, equal volume of each serial 2-fold dilution of antisera was mixed with virus suspesions containing approximately 100 $TCID_{50}$ in 0.1 ml per roller tube or in 0.025 ml per microplate well. The mixtures were incubated at 37 °C for 30 min. Four to six cultured tubes or microplate wells were used for each serum dilution. Cytopathic effect (CPE) was observed at days 5 to 7 and the NT titer was expressed as a reciprocal of the serum dilution which showed 50% CPE calculated by the method of Reed and Muench. The grouping-NT was performed in MK cell cultures by mixing of approximately 100 $TCID_{50}$ of each test virus with an equal vol of immune sera which contained approximately 50 neutralization antibody units. The mixture was incubated at 37 °C for 30 min and then was inoculated into 4—6 culture tubes or microplate wells. The cultures were held for 5 to 7 and the CPE was determined.

Haemagglutination and haemagglutination-inhibition tests. Haemagglutination (HA) and haemagglutination-inhibition (HI) tests were performed by the microtiter method. HI test was carried out with guinea pig red blood cells (RBC) and the sedimentation patterns were read at 4 °C.

Electron microscopy. The virus grown with MK cells was viewed in the electron microscope. The materials of 12005T, 12390L and 12012B viruses were concentrated to 50—100-fold by differential and ultracentrifugations at velocities of 1,000 and 100,000 g, respectively. The materials of 12337N and 12008T were prepared as described by Valentine and Pereira (1965). Photographs were taken by HU-11B (Hitachi) electron microscope after negative staining with 2% uranyl acetate.

Virus infectivity titration and passaging. Three kinds of cell lines grown in roller tubes were used to assay the growth properties of viruses. Infectivity was titrated by inoculation of 0.1 ml of each serial 10-fold dilutions of the virus suspensions into four tubes. Inoculated cell cultures were observed daily for the appearance of CPE, and medium was replaced every 4 to 5 days. CPE was scored between 14 and 21 days after inoculation. Titres were expressed as $\log_{10}$ $TCID_{50}$ per 0.1 ml as calculated by the method of Reed and Muench (1938). Upon appearance of CPE, the infected cultures and their medium were frozen and thawed three times to disrupt the cells. The suspension was clarified by centrifugation at 3,000 rev/min for 20 min, then the supernatant was used for passage. This procedure was repeated several times for consecutive transfers.

Results

Virus isolations and grouping of unidentified viruses

Eight of parainfluenza type 3 viruses, 10 of SV5, 4 of Murayama viruses and a total 23 strains of unidentified viral agents were isolated from 73 specimens of 53 cynomolgus monkeys suffering from respiratory diseases. All specimens were inoculated in MK cell cultures and embryonated hen's eggs, and a part of them was also inoculated in HEp-2 cell cultures by the method described previously (Nishikawa *et al.*, 1974). Unidentified viruses were isolated by MK and HEp-2 cell cultures, and all of them showed a mar-

Table 1. Cross NT of group 1 viruses

Virus	Antiserum	
	12005T	12390L
12005T[1]	512[4]	8
12216N[2]	32	4,096
12243N	<4	≧4,096
12344N	11	2,048
12390L[3]	<4	≧4,096

1) Virus isolated from a cynomolgus monkey throat swab.
2) Virus isolated from cynomolgus monkey nasal secretion.
3) Virus was isolated from lung tissue of a dead cynomolgus monkey.
4) Titres represent reciprocals of serum dilution.

Table 2. Grouping NT of group 1 viruses

Virus	Antiserum	
	12005T	12390L
12005T*	0/4**	4/4
12340N*	0/4	4/4
12216N	3/4	0/4
12219N	4/4	0/4
12231L*	2/4	0/4
12243N	4/4	0/4
12332T	1/6	0/6
12344N	1/4	0/4
12344T	4/4	0/3
12352L	4/4	0/4
12383L	4/4	0/4
12390L	4/4	0/4
12396L	4/4	0/4

* Symbols are the same as Table 1.
** Number of cultures occured CPE/Number of cultures tested.

ked CPE in the inoculated cell cultures. Each virus isolated in HEp-2 cells was also isolated to MK cells. Among them, 6 viruses showed HA with chick and guinea pig RBC.

The unidentified isolates were divided into three groups, based upon the characters of their CPE in MK cell cultures and upon their HA activities. Group 1 virus, including 13 isolates, generally showed marked CPE resembling enterovirus-CPE characterized by the production of round cells. The CPE resulted in the destruction of monolayer cells within 1 to 3 days. Among them, strains 12005T and 12340N caused a complete destruction of monolayers. The other eleven strains also showed a marked CPE,but a part of cells still adhered to the tube walls. Neither virus of this group agglutinated chick or guinea pig RBC. Group 2 was composed of four strains which failed to agglutinate chick or guinea pig RBC. Each virus of group 2 showed adenovirus-like CPE, that is round and grape-like clusters appearing within four to five

Table 3. Grouping NT of group 2 viruses

Virus	Antiserum		
	12005T	12390L	12337N
12005N*	4/4**	4/4	0/4
12016N	4/4	4/4	0/4
12036T**	4/4	4/4	0/4
12337N	4/4	4/4	0/4

* Symbols are the same as in Table 1.
** Numbers are expressed as in Table 2.

Table 4. Cross NT and HI test of group 3 viruses

Virus	Antiserum			
	NT		HI	
	12008T	12012B	12008T	12012B
12008T	4,096	<4	1,024	<16
12012B**	<4	710	<16	1,024
12337T	<4	128	<16	2,048
12339N*	4	512	<16	2,048
12339T	<4	128	<16	1,024
12340T	<4	128	<16	2,048

* Symbols are the same as in Table 1.
** This virus was isolated from trachea and bronchus of a dead cynomolgus monkey.

days in the gradually degenerating monolayers. Group 3 was composed of 6 strains which agglutinated chick and guinea pig RBC at 4°C but failed to agglutinate them at room temperature. The cells became round and intensely granular and tended to form grape-like clusters within 1 to 3 days, a pattern resembling adenovirus CPE.

In some cases, different viruses were isolated from nasal secretion and throat swab of the same animal, respectively, or two different viruses were isolated from the same sample of an animal. For example, group 1 and 3 viruses were isolated from a nasal and a throat sample of the same monkey (No. 12340), respectively. A group 1 virus and parainfluenza type 3 virus were isolated from a nasal sample of the monkey No. 12219.

Cross NT and HI tests

To compare the antigenic relationship of these unidentified viruses with each other, cross NT among group 1 viruses were performed. The results are shown in Table 1. Antiserum against 12005T had a homologous titre of 512, but it revealed only minor cross-reactivity with 12216N and 12344N, and showed no neutralizing activity to 12243N and 12390L. In contrast, antiserum against 12390L showed a homologous titre 4,096 or greater and had a titre of 2,048—4,096 or greater against the other group 1 viruses except 12005T. It could be seen from these data that group 1 viruses could be serologically divided into two subgroups. The grouping NT was carried out for all 13 virus strains. As shown in Table 2, 12005T and 12340N strains were completely neutralized with 12005T immune serum, but the other 11 strains were not neutralized or partially neutralized by this serum. On the other hand, both 12005T and 12340N strains were not neutralized with 12390L immune serum, but the other 11 strains, including homologous 12390L strains, were completely neutralized by this immune serum.

Table 3 shows grouping NT of group 2 virus strains. All four strains were not neutralized with antisera to 12005T or 12390L of group 1, but were

Table 5. Growth spectrum of group 2 and 3 viruses in continuous cell cultures

Group	Virus strain	Original material		Virus titre after passage in cells		
		Number of passages in MK cell	Virus titre	JINET	R-66	Vero
2	12005N*	6	7.5[1]	3.5[1] (5)[2]	2.9 (8)	≧2.0 (3)
	12016N	6	≧6.5	3.5 (6)	4.0 (8)	5.7 (6)
	12036T*	7	5.8	3.5 (3)	2.0 (4)	2.8 (7)
	12337N	9	6.5	≧3.5 (3)	2.0 (6)	3.3 (7)
3	12008T	8	6.5	1.0 (4)	3.7 (5)	<1.0 (3)
	12012B*	4	7.0	<1.0 (3)	<1.0 (3)	≧3.5 (3)
	12337T	3	≧8.5	1.0 (4)	2.7 (5)	2.6 (3)
	12339N	4	≧8.5	2.2 (3)	2.7 (5)	5.3 (3)
	12339T	3	≧8.5	<1.0 (3)	4.6 (3)	3.5 (3)
	12340T	3	7.8	2.2 (3)	2.5 (3)	1.5 (3)

* Symbols are the same as in Table 1.
[1] $\log_{10}$ $TCID_{50}$ /0.1 ml
[2] Number of passages made.

completely neutralized by the immune serum against 12337N which was the representative strain of group 2 viruses. Therefore, there was no antigenic relationship between the viruses of groups 1 and 2, and the four strains were judged to belong to the same group.

As regards the 6 strains of group 3 (Table 4), cross reactions were hardly found between 12008T and the other 5 viruses. Naturally, 12008T antiserum had a high titre to the homologous virus (1 : 4,096 in NT; 1 : 1,024 in HI), but it showed no NT or HI titres to any heterologous virus. In contrast, 12012B antiserum showed NT or HI titres to each heterologous virus, except of 12008T, the levels being nearly equal to those with homologous virus (710—128 in NT; 2,048—1,034 in HI). These data suggested that group 3 viruses could be divided into two serological subgroups.

Electron microscopy

Electron micrographs of 12005T and 12390L viruses belonging to group 1 demonstrated their icosahedral shape about 35 nm in diameter. Morphological examinations of viruses 12337N from group 2, and of viruses 12008T and 12012B from group 3 showed that they both had a shape of icosahedron about 90 nm in diameter (Fig. 1).

Growth spectrum of the viruses in some continuous cells

The clarified supernatants of each MK cell culture infected with ten strains of group 2 and 3 were inoculated into three continuous cell cultures, such as JINET, R-66 and Vero cells and were serially passaged. The results are given in Table 5. In general, all four strains of group 2 grew well and could

Table 6. Results of the grouping of unidentified viruses

Group	CPE in MK cells	HA	Morphology (Representative strain)	Subgroup by cross NT	Strain	Identification by Dr. Kalter's laboratory
1	Rounding, degenerated within 1—3 days	Negative	Icosahedron, about 35 nm in diameter (12005T) (12390L)	A	12005T 12340N	N.D.*
				B	12216N, 12219N 12231L, 12243N 12332T, 12344N 12344T, 12352L 12383L, 12390L 12396L	
2	Grape-like cluster appeared within 4—5 days	Negative	Icosahedron, about 90 nm in diameter (12337N)		12005N, 12016N 12036T, 12337N	SV25 SV34
3	Rounding and grape-like cluster appeared within 1—3 days	Positive for chick and guinea pig RBC at 4 °C	Icosahedron, about 90 nm in diameter (12008T) (12012B)	A	12008T	SV23
				B	12012B	SV17
					12337T	V340
					12339N, 12339T	SV15
					12340T	SV31

* Not done

be serially transferred in each of three cell cultures. On the other hand, some virus strains of group 3 grew poorly or could not be serially transferred in R-66 or Vero cells, and the majority of strains did not grow in JINET cells. Growth of the viruses in these cell cultures was slower than that in MK cells. The CPE produced by these viruses were round and grape-like clusters. Virus yields in each group with each cell line were analysed in more detail.

Group 2 viruses

Four strains of this group grew in JINET and Vero cells; all these strains grew also in R-66 cells, however, the virus titres of 3 strains, with the exception of strain 12016N, were slightly lower than in JINET cells. The virus titre of strain 12016N in Vero cells was higher than that of other virus strains. CPE in three cell lines has developed within 4 to 8 days after infection.

Group 3 viruses

We experienced difficulties with the growth and maintenance by serial passages of the strains 12008T, 12012B, 12337T and 12339T in JINET cells. All group 3 viruses, with the exception of the 12012B strain, grew well in R-66 cells. The strain 12008T grew in R-66 cells but failed to replicate in

JINET and Vero cells after 3rd or 4th passages. All group 3 strains, except of strains 12008T and 12340T grew in Vero cells. Concerning the 12012B strain, none of JINET and R-66 cells tested was found to support its growth, Vero cells being the only susceptible cell line. The CPE appeared within 4 to 8 days after infection in all three cell lines.

Discussion

The presented results are summarized in Table 6. From the findings we judged that group 1 viruses are enteroviruses, while group 2 and 3 viruses were classified as adenoviruses, referring to the results of several other studies (Archetti *et al.*, 1961, 1963; Heberling *et al.*, 1965, 1972*a*, *b*; Rapoza, 1967; Kim *et al.*, 1967; Hull, 1968; Eugster *et al.*, 1969; Basnight *et al.*, 1971; Kalter, 1973, 1982). Ten strains belonging to groups 2 and 3 were sent to Dr. S. S. Kalter, NIH and to the WHO Collaborating Center for Reference and Research in Simian Viruses, San Antonio, Texas and all of them were found simian adenoviruses. The results of the identification are also shown in Table 6.

Let us compare the grouping of our isolates with the properties of the prototype strains of the same serotype as described in other reports. In this study, 10 strains of adeno-like isolates were divided into groups 2 and 3 according to their HA characters, and then the group 3 viruses were subdivided into subgroup A and B according to their serological interrelationships. In Dr. Kalter's laboratory, out of 4 strains of group 2, two were identified as SV25, and another 2 as SV34. The strain of subgroup A in group 3 was identified as SV23, and out of 5 strains of subgroup B, 2 were identified as SV15, and the other 3 were SV17, SV31, and V340, respectively (Table 6). From this table it can easily be seen that, except 12337T (V340), our groups 2 and 3 just correspond to groups III and II of Rapoza, respectively (Rapoza, 1967). We found serological relationships by grouping-NT among the viruses in the group 2 (Table 3), although there was no interrelationship between SV25 and SV34 in the previous reports (Rapoza, 1967; Hull, 1968). We could not demonstrate interrelationships between strains of subgroups A and B (group 3) but close relationships were observed among the subgroup B strains (Table 4). Interrelationships were constantly demonstrated among SV15, SV17 and SV31 by NT and HI test, whereas, SV23 showed no serological relationship to above three serotypes. In short, our grouping of the ten viruses into two groups and our data of serological relationships among the viruses in group 3 are in good agreement with other reports, although some discrepancies are seen in the serological relationships between our data on group 2 viruses and those of Rapoza's group III viruses. However, these discrepancies seem to account either for individual differences of immunized animals or due to the methods of immunization or both, as also stated by others (Hull, 1968). There was no information about the grouping of V340 so far, but we have demonstrated that the V340 serotype belongs to group II of Rapoza, and that it has some antigen common with each SV15, SV17 and SV31 serotypes (Table 4).

It is suggestive that V340 was isolated from a cynomolgus monkey in this study, because no report has been published concerning the isolation of V340 from this animal species, although the evidence of antibody conversion against V340 has been presented with various species of New and Old world monkeys including the cynomolgus monkey (Kim *et al.*, 1967; Eugster *et al.*, 1969, Kalter, 1973). We isolated not only V340 (strain 12337T) from a throat swab of a cynomolgus monkey suffering from mild respiratory disorders but also SV34 (strain 12337N) from a nasal swab of the same animal. Morevoer, Murayama virus (strain 12337 NT) was isolated from the mixture of nasal and throat swabs (Nishikawa *et al.*, 1976). There were some other cases in which more than one virus was simultaneously isolated either from the same sample or from different samples coming from the same animal. So far, it is not clear whether one of such viruses was pathogenic and whether the other agent was present in a latent state in the animal.

Frequent appearance of latent viruses such as foamy virus or SV5 in simian primary cell cultures is a serious problem in virus research since introduction of cell cultures (Hooks *et al.*, 1975; Yoshida *et al.*, 1965). An established cell line is generally much easier to culture than a primary culture, but only a few information is available on sensitivity of cell lines to simian viruses (Tsuchiya *et al.*, 1963; Heberling *et al.*, 1965; Hillis *et al.*, 1969; Darai *et al.*, 1980; Niskihawa *et al.*, 1981).

In our laboratory, we routinely use three kinds of cell lines, such as JINET R-66 and Vero for virus isolation. Thus we tested the sensitivity of these cell lines to the isolated viruses. The present report describes the preliminary experiments designed to compare the yield of 10 isolated strains of simian adenoviruses in three cell lines. As can be seen in Table 5, different species of simian adenoviruses showed a considerable difference in their ability to grow in these cell cultures. Viruses of different groups also showed different growth characters, that is, the viruses of group 2 grew well in JINET cells in which the viruses of group 3 grew very poorly. This suggests that certain biological characteristic of the virus may be associated with its HA activity. Rapoza reported that the replication of SV36 in LLC-MK_2 cells was inhibited by the adeno-associated virus, so it will be interesting to follow the virus yields of other serotypes in the given cell lines.

Identification of enterovirus-like isolates and the study of their biological characters and pathogenicity in monkeys has remained unsettled so far.

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Explanation of Electron Micrographs (Plates XLIV—XLV):

Fig. 1. Electron micrographs of negatively stained simian viruses.
I — 12005T, III — 12337N, V — 12012B,
II — 12390L, IV — 12008T.
Bar represents 50 nm.