Taxonomic Features of Seven Serotypes of Simian and Ape Foamy Viruses

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Two new simian viruses were recovered from squirrel monkeys and galagos. They possess foamy virus group properties which appear distinct from other virus groups. Serologically they were designated types 4 and 5 and are completely specific from each other and from types 1, 2, 3, 6, and 7 by neutralization tests. The study includes a comparison of the properties of all seven types in one laboratory.

The simian foamy viruses are an as yet incompletely characterized group of viruses; their characteristics include production of a distinctive cytopathic effect (CPE) with giant cell (syncytium) formation, absence of inclusion body formation, lack of pathogenicity for common laboratory animals, absence of hemagglutinin, and sensitivity to lipid solvents. Although these are not definitive taxonomic features, they serve in separating these viruses from other groups. Identification and classification are accomplished chiefly by neutralization with prototype antisera. The foamy viruses have been reviewed recently by Rogers et al. (10), Hull (6), and Wilner (13).

This report describes the isolation of two new serotypes, types 4 and 5, from squirrel monkeys and African bush babies (galagos), respectively, and compares the serological and biological properties of the seven simian foamy viruses.

MATERIALS AND METHODS

Cells. Primary rabbit kidney (P-RK) cells were used for virus isolation, titration, and neutralization tests except where noted otherwise. They were prepared from 1- to 2-lb. (453.5 to 907.1 g) white New Zealand rabbits and grown at 36 C in Eagle's basal medium (Hanks) with 10% calf serum; for maintenance, the serum was reduced to 5% (v/v) and sodium bicarbonate was increased 3.7-fold to 0.123% (w/v). BHK-21 clone 13 cells were received from Flow Laboratories, Inc., Rockville, Md.

Viruses. Viruses employed were the ATCC prototype strains of foamy virus type 1 (FV21), type 2 (FV34) of Johnston (7), and type 3 (FV2014) of Stiles et al. (11). Strains 1224 and 1557 are selected as "prototypes" for types 4 and 5. Type 6 (CV-1, Pan-1) and type 7 (CV-11, Pan-2) were described by Rogers et al. (10). Strains from Stiles and from Rogers were generously provided prior to their publications. Virus pools were prepared after prolonged incubation when CPE attained +++, by scraping cells into the fluid, lysing in the VirTis homogenizer at 45,000 rev/

min for 2 min at 4 C (4 min for larger volumes of 40 to 200 ml), and clarifying by centrifuging at 1,800 × g for 20 min at 4 C. Virus titrations consisted of tenfold dilutions in quadruplet tube cultures which were incubated for 1 month, and results were expressed as tissue culture infectious doses $(TCID_{50})/0.2$ ml. Chloroform lability was tested by the procedure described by Feldman and Wang (3). The tests for pH lability and heat lability were done as described by Hamparian et al. (5). Methods of foamy virus serum-neutralization tests in P-RK cells were reported (7).

Animal inoculation. The suckling hamsters and mice were less than 24 hr old when inoculated with cell-free virus preparations which had been centrifuged twice at 1,800 × g at 4 C and filtered through membrane filters (450-nm porosity; Millipore Corp., Bedford, Mass.). Intracerebral and subcutaneous (dorsal between the scapulae) inoculations of 0.03 ml were used. For preparation of reference antisera, horses were inoculated with types 1, 2, and 4; rabbits were used for types 3, 5, 6, and 7.

RESULTS

Isolation of types 4 and 5. Seventeen squirrel monkeys (*Saimiri sciureus*) were procured from three suppliers (Tarpon Zoo, Tarpon Springs, Fla., and two local pet shops) to conduct transmission tests with the types 1, 2, or 3 foamy viruses in this relatively inexpensive primate. Unfortunately, they were unsuitable since they were found (11 Jan. 1965) to contain a serologically unrelated foamy virus (type 4) which grossly resembles type 1, 2, and 3 by CPE and absence of inclusion bodies. The data of Table 1 show that inoculation of throat swabs from the monkeys 1, 2, and 3 elicited giant cells in P-RK cultures within 7, 15, and 22 days, respectively. In addition, viruses were recovered from the kidneys of animals 2 and 4 by employing the "unmasking" procedure in which the animal's own kidney cells were trypsinized and cultured. Each of these

TABLE 1. *Foamy viruses present in throat swabs from squirrel monkeys and two species of bush babies and occurrence of neutralizing antibody in their sera*

Animal	Throat swab and days of incubation for CPE[a]		Serotype of virus isolate	Neutralizing titer of each animal's serum against prototype virus				
				Type 1	Type 2	Type 3	Type 4	Type 5
Saimiri sciureus								
1	+	7	4 (1224)	0[b]	0	0	16	0
2	+	15	4	0	0	0	64	0
3	+	22	4	0	0	0	64	0
4	−		4 (kidney)	0	0	0	0	0
5	+	26		0	0	0	16	0
6	+	22		0	0	0	0	0
7	+	30		0	0	0	0	0
8	−			0	0	0	0	0
Galago crassicaudatus panganiensis[c]								
1	+	8	5	0	0	0	0	8
2	+	12	5 (1557)	0	0	0	0	64
3	+	12	5	0	0	0	0	8
4	+	20	5	0	0	0	0	16
5	−			0	0	0	0	8
6	−			0	0	0	0	0
Galago senegalensis braccatus[c]								
1	−			0	0	0	0	—
2	−			0	0	0	0	—
3	−			0	0	0	0	—
4	−			0	0	0	0	—
5	−			0	0	0	0	—
6	−			0	0	0	0	—

[a] Cytopathic effect.

[b] Symbols: 0, less than 1:8; —, indicates not tested.

[c] Bush baby specimens shipped in dry ice were generously provided by Robert W. Cooper from his animals, on the National Cancer Institute contract PH 43-63-56 at his laboratory at the Institute for Comparative Biology of the Zoological Society of San Diego, Calif.

eventually developed CPE with giant cells. Subculture of the viruses from the above four animals gave typical CPE and complete neutralization by 4 units of antibody from monkey 1 (from whose throat swab the prototype-4 strain 1224 was isolated on two occasions). These viruses were also neutralized by 16 units of antibody prepared in either of two horses after prolonged immunization with live strain 1224. Cultures of throat swabs from monkeys 5, 6, and 7 were at first thought to be definitely positive with only one or two giant cells; however, subcultures were without CPE. Only one of these three monkeys contained antibody, whereas three of the above four (no. 1 to 4), which definitely contained subculturable virus, had homotypic neutralizing antibody ranging in titer from 1:16 to 1:64. All eight Saimiri monkeys were without antibody to types 1, 2, 3, and 5. Eleven additional squirrel-monkey sera were generously provided by D. C. Gajdusek of

the National Institutes of Health. Although they had been inoculated with Kuru patient's specimens, the finding of type 4 neutralizing titers of $\geqq 1:8$ in 9 of these 11 sera is taken to indicate natural foamy-virus infection.

The remainder of Table 1 shows the type 5 foamy virus isolation on 14 June 1966 from four of six "greater bush babies" (*Galago crassicaudatus panganiensis*), and the bottom of the table shows absence of foamy virus in all six of the "lesser bush babies" (*Galago senegalensis braccatus*). Neutralizing antibody to prototype-5 virus strain 1557 was present at titers of 1:8 to 1:64 in each of the four virus-positive animals. All 12 African bush babies were devoid of neutralizing antibody when tested against types 1, 2, 3, or 4 prototypes.

The four isolates (type 5) were identified first by complete neutralization with 4 units of antibody from animal 2 (whose throat swab had

yielded the prototype virus 1557). Later, after preparing rabbit antisera against strain 1557, these four isolates were again identified as type 5, since 16 units of these antisera completely neutralized each isolate.

Immunology. For comparison to other serotypes, each virus was adapted from the P-RK cells to a cell line to minimize contamination with in-

digenous rabbit viruses, as follows: types 1 and 2 KB, type 3 LLC-MK2 and KB, type 4 BHK-21/13, types 5, 6, and 7 Hela. The viruses often grew more slowly with delayed appearance of giant cells in cell lines as compared to primary monkey kidney cells. Suspension cultures in Spinner flasks (Bellco Glass, Inc., Vineland, N.J.) were used for production of pools of types 1 and

TABLE 2. *Serum neutralizing antibody titers after immunizing horses or rabbits with live foamy virus types 1 to 7*

Animal immunized	Serum type	Animal no.[a]	No. of injections	Time since first injection (weeks)	Antigen neutralized[b]						
					Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7
Horse	1	7C	5	5	64	0	4	0	0	0	0
		7E	7	12	256	0	8	0	0	0	0
		8C	6	9	32	0	4	0	0	0	0
Horse	2	2A	0		0	0	0	0			0
		2G	5	14	0	1,024	4	4	0	0	64
		3A	0		0	0	0	0			0
		3D	6	13	0	512	0	0	0	0	32
Rabbit		480E	4	10	0	128					64
		1368	4	9	0	512					64
Rabbit	3	33B	4	8	0	0	64	0		0	0
		34B	4	8	4	0	256	0	0	0	0
		35B	4	8	0	0	256	0	0	0	0
		36B	4	8	0	0	>256	0	0	0	0
Horse	4	4C	6	10	0	0		16–32			
		4D	7	14	0	0	0	32	0	0	0
		6C	6	9	0	0		16–32	0	0	0
		6D	7	13	0	0	0	64	0	0	0
Rabbit	5	51D	4	8	0	0	0	0	256	0	0
		52D	4	9	0	0		0	128	0	0
		53D	4	9	0	0	0	0	1,024	0	0
		54D	4	9	0	0		0	32	0	0
Rabbit	6	71B	4	8	0	0	0	0	0	256	0
		71D	4	16					0	4,096	
		72B	4	8	4	0	0–4	0	0	64	0
		73B	4	8	0	0	0–4	0	0	64	0
		73D	4	16						>256	0
		73E	4	18						>256	0
		73G	5	27						>256	0
		74B	4	8	0	0	0	0	0	64	0
Rabbit	7	61B	4	8	0	0	0			0	128
		62B	4	8	0	0	0	0		4	32
		63B	4	8	0	4	0	0	0	0	128
		64B	4	8	0	0	0	0	0	0	64
		64E	4	19						0	>256
		64H	5	30						0	>256
		64I	5	37						0	>256

[a] Symbols: A, preimmune serum; B, C, D, etc., successively collected postimmune.

[b] 0, Titers of less than 1:4. All preliminary sera of horses and rabbits had homologous titers of zero, except 1:4 titers occurred with three of the four rabbits used for type 3.

2 since they could be cultured for long periods without a difficulty which occurred in older monolayer cultures, namely loss of infected cells because they detached from the monolayer (particularly, infected giant cells). These cultures received standard Eagle's minimal essential medium for suspension cultures; two or three times weekly, one-third of the volume was harvested for preparation of virus pools and replaced with fresh medium. Virus yields were $10^{4.0}$ and $10^{5.0}$ TCID$_{50}$/0.2 ml, respectively, when titrated in P-RK.

The virus pools from the above cell lines were used as antigens for the neutralization tests shown in Table 2. The reference antisera listed in this table were prepared as follows. Horses were immunized with types 1 and 2 by a series of three, weekly, intramuscular inoculations of 25 ml of live aqueous virus suspensions which were followed with two, similar, bimonthly inoculations. Two weeks later another inoculum of 20 ml in Freund's incomplete adjuvant was administered in five intramuscular sites (4 ml each). This was repeated 1 to 2 weeks later. A similar regimen was employed with the relatively weak type 4 virus, except that the virus preparations consisted of fourfold concentrates in Hanks solution prepared by decanting the monolayer cultures and scraping the infected cells into one-fourth of the culture's original volume, before lysing in the VirTis homogenizer. Rabbits were employed for antibody production with types 3, 5, 6, and 7.

Two, weekly, aqueous, 10-ml virus inoculations were given intravenously and followed 7 to 10 days later with 10-ml intramuscular inoculations of the adjuvant preparations with 2.5 ml in each of four sites. The adjuvant inoculations were repeated 7 to 10 days later. Serial serum harvests were taken at the time intervals listed in column 5 of Table 3, the main data consisting of sera taken 8 to 14 weeks after the first immunization. Late booster inoculations with aqueous virus preparations were given to rabbits no. 73 and 64 as shown. The kinds of host cells and number of passages of virus used for immunization, together with virus titers in P-RK cells (TCID$_{50}$/0.2 ml) of the final passage used as inoculum, were as follows: type 1 in P-RK 13, KB 1, LLC-MK$_2$ 6, $10^{4.0}$; type 2 in P-RK 22, KB 1, $10^{5.0}$; type 3 in P-RK 18, LLC-MK$_2$ 6, $10^{4.7}$; type 4 in P-RK 20, 10^3; type 5 in P-RK 3, HeLa 7, 10^3; type 6 in P-RK 6, $10^{3.5}$; and type 7 in P-RK 6, $10^{4.0}$.

Both of the horses immunized with type 1 simian foamy virus developed fair homologous antibody cross-reacting only with type 3. This was 32-fold less than the homologous titer of 1:256 in horse 7. Occasional 1:4 cross-reactions appear throughout the table, and it is thought that they do not detract from type specificity since the same situation occurs, for example, among human adenovirus or myxovirus serotypes. Each of the horses immunized with type 2 strongly cross-reacts against type 7, with titers only 16-fold less

TABLE 3. *Properties of seven serotypes of foamy viruses*[a]

Properties	Virus type						
	1	2	3	4	5	6	7
Ether sensitivity							
Ether-treated virus titer	10	10	10	0			
Control virus titer	$10^{3.0}$	$10^{3.0}$	$10^{4.0}$	$10^{3.3}$			
Chloroform sensitivity							
Chloroform-treated virus titer	0	0	0	0	0	0	0
Control virus titer	$10^{4.5}$	$10^{4.5}$	$10^{4.5}$	$10^{3.3}$	$10^{3.5}$	$10^{5.7}$	$10^{4.5}$
pH sensitivity							
pH 3.0, 2 hr, virus titer	0	0	0	0	0	0	0
Control virus titer	$10^{4.3}$	$10^{4.3}$	$10^{3.3}$	$10^{3.5}$	$10^{3.5}$	$10^{5.7}$	$10^{4.5}$
Heat sensitivity							
50 C, 30 min, virus titer	10^0	0	0	0	0	10^0	0
Control virus titer	$10^{2.2}$	$10^{4.5}$	$10^{2.0}$	$10^{3.5}$	$10^{3.5}$	$10^{5.7}$	$10^{4.5}$
Inclusion body	−	−	−	−	−	−	−
Large giant cells	+	+	+	+	+	+	+
Hemadsorption	−	−	−	−	−	−	−
Hemagglutination	−	−	−	−	−	−	−
Suckling mouse pathogenicity	−	−	−	−	−	−	(±)
Titer of virus inoculated into suckling mice and used in hemagglutination test	$10^{4.7}$	$10^{4.0}$	$10^{4.7}$	$10^{4.0}$	$10^{3.5}$	$10^{5.7}$	$10^{4.5}$

[a] Virus titers are listed as TCID$_{50}$/0.2 ml after culturing in primary rabbit kidney cells for 4 weeks.

than the homologous titers. In addition, type 2 antisera from each of two rabbits cross-reacted even more against type 7. It is unlikely that these animals were immunized with a mixture of viruses, since the sera were prepared before type 6 or 7 virus were received in this laboratory. This is only a one-way cross-reaction, since the four type 7 rabbits were essentially negative to

type 2 even though two had excellent homologous titers (1:128).

The sera from each of the animals immunized with types 3, 4, and 5 were completely type specific. The findings of Rogers et al. (10) that type 6 and 7 did not cross-react with each other are confirmed with each of four rabbits used for either type. Seven of these eight animals yielded titers of
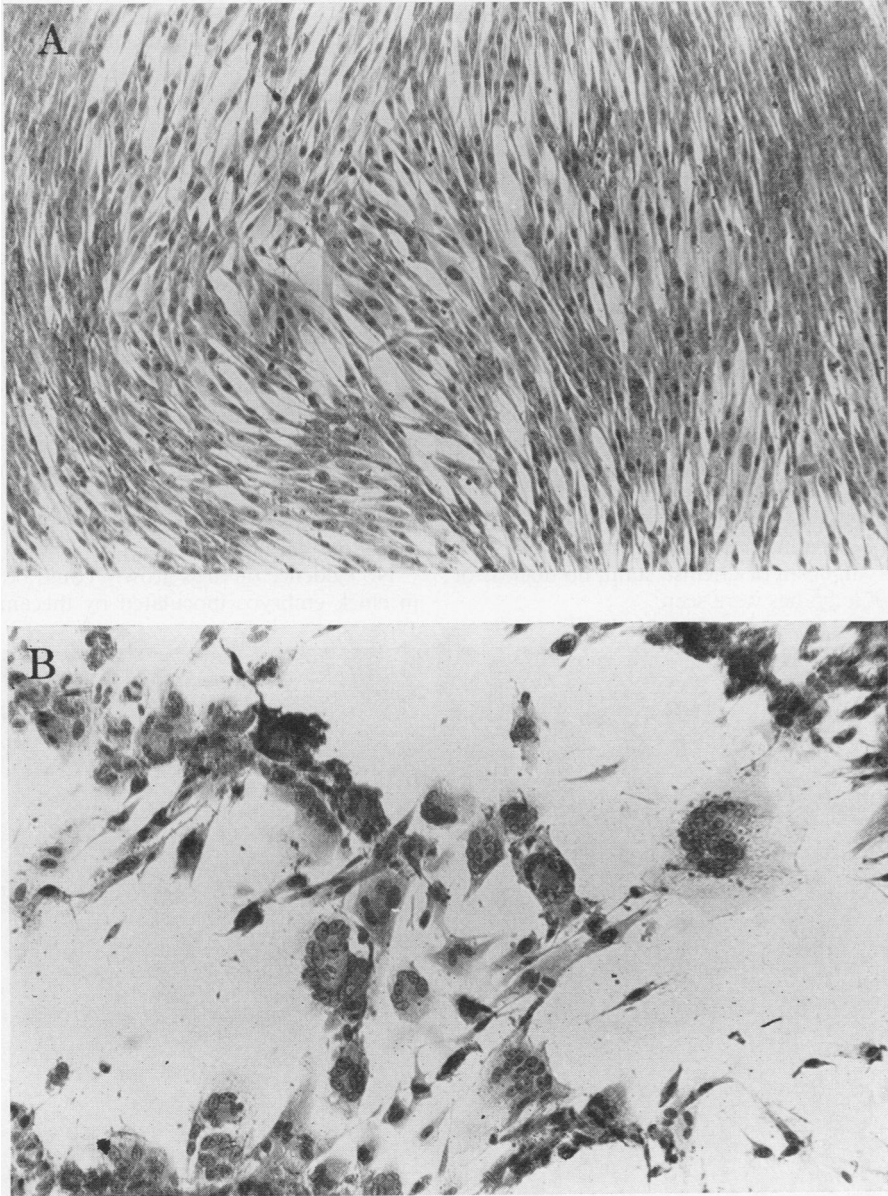
FIG. 1. *Giant cell (syncytium) formation by foamy virus serotype 4 strain 1224. (A) Uninfected BHK-21/13 cells; (B) BHK-21/13 cells 7 days after infection with $10^{4.7}$ TCID$_{50}$ of foamy virus type 4 (primary rabbit kidney 22, BHK-11). Cytopathic effect involved about two-thirds of the cells. Giemsa stain × 912.*

1:64, or greater, and were without significant cross-reaction to the other serotypes.

Viral characterization. Certain taxonomic properties of foamy viruses types 4 and 5 are compared to the other serotypes in Table 3. All were sensitive to the lipid solvents, indicating presence of a viral envelope; chloroform caused complete inactivation, whereas diethyl ether treatment at 4 C for 18 hr caused at least 100-fold decrease in titer to 1:10 for types 1, 2, and 3. Controls of influenza A1 or Newcastle disease virus were completely inactivated by ether. Complete loss of infectivity occurred on exposure to pH 2.7 to 3.0 for 3 hr at 25 C in tris(hydroxymethyl)aminomethane-hydrochloride buffer. Each was completely inactivated by 50 C for 30 min, except for trace amounts of residual virus with types 1 and 6.

Hemadsorption and hemagglutination tests were negative with various red cells from group 0 humans, rhesus, rabbit, guinea pig, hamster, chick embryo, and newborn or adult chicken at 4, 25, or 37 C. For the hemadsorption tests, virus-infected P-RK monolayers were washed free of calf sera and replaced with Eagle's BME without serum.

Inclusion bodies were intensively searched for to aid in distinguishing from paramyxovirus or herpesvirus. P-RK and at least one cell line were used for each virus. After using several fixatives such as Zenker's with acetic acid, Bouin's, 10% buffered Formalin, or methanol and staining with hematoxylin-eosin or Giemsa stain, no nuclear or cytoplasmic bodies were seen.

Giant cells were elicited by all 7 types; photomicrographs of giant cells induced by foamy virus types 4 and 5 are seen in Fig. 1. The CPE induced by type 4 in BHK-21/13 cultures (Fig. 1B) consisted of many giant cells (10 to 30 nuclei) with more retraction from the monolayer than found with other types. BHK-21/13 produced in general higher titers of foamy virus but *less* fusion than did other cells. Type 5 was the only serotype repeatedly negative in BHK-21/13.

The giant-cell CPE produced by type 5 in HeLa cells (Fig. 2) is indistinguishable from that produced by types 1, 2, 3, 6, and 7, except that type 5 never progresses beyond + +, whereas the others commonly develop to + + + but rarely + + + +.

Lack of animal pathogenicity. Virus types 1 and 2 were each inoculated into 5 litters of suckling, Syrian, golden hamsters. No obvious disease or oncogenesis was seen within 15 months. In addition, each of the seven virus serotypes was inoculated into 5 litters of random-bred suckling Swiss mice (Table 3). The only possibly positive results after 24 months of observation were in a single litter of 10 inoculated with type 7, in which two females developed dorsal subcutaneous tumors near the site of inoculation between 12 and 15 months after inoculation. One tumor is adapted to cell culture; no giant cells are seen. Transplantation was unsuccessful in two lines of mice, perhaps because the animals with tumors were from a random-bred colony.

No evidence of virus growth could be obtained in chick embryos inoculated by the amniotic or
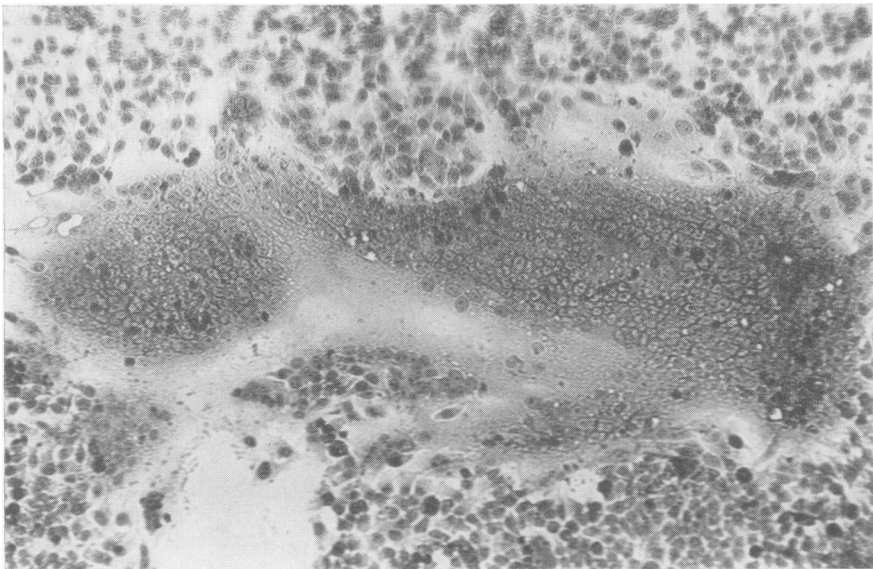

FIG. 2. *Giant cell formation in Hela cells by foamy virus serotype 5 (primary rabbit kidney 3, Hela 10); 7th day after infection with $10^{3.7}$ TCID$_{50}$. + + cytopathic effect. Giemsa stain $\times$ 912.*

allantoic route with types 1, 2, 3, 4, and 5 virus and harvested after 3 days. Amniotic and allantoic fluid harvests were negative for hemagglutinin and noninfective in P-RK cells.

The live viruses administered intramuscularly for antibody production in horses or rabbits did not elicit obvious disease. This included two horses each for types 1, 2, and 4, and one horse for type 3; four rabbits each were negative with types 3, 5, 6, and 7 and two others for type 4.

Further absence of pathogenicity was found when inoculating a single capuchin monkey (*Cebus apella*) with type 5 virus. This monkey was without type 5 neutralizing antibody at onset. Virus (2 ml) titering $10^{8.7}$ TCID$_{50}$ per 0.2 ml was inoculated intraperitoneally, 2 ml into the throat, and 0.2 ml was dropped into each nostril. Plasma specimens were virus-negative on day 0, 2, 3, 6, 8, 10, and 16 when tested in P-RK cells. Homogenized (45,000 rev per min per 2 min) "buffy coat" specimens at each time interval were also negative. Throat swabs were virus-negative on day 16. An animal handler was bitten deeply on the hand on day 6 but fortunately healed quickly and is without obvious disease.

DISCUSSION

The findings appear remarkable in that natural foamy virus infections seem quite host-specific; rhesus monkeys have been reported, so far, to harbor only type 1 (6, 12); the Formosan monkey, types 1 and 2 (7); African green monkeys, types 1, 2, and 3 (11); squirrel monkeys, type 4; galagos, type 5; and chimpanzees, types 6 and 7 (10). The galago virus is of further interest since it is the first viral isolate, of any kind, from a prosimian species.

Further evidence that the newly described types 4 and 5 are not common human viruses was obtained by finding absence of neutralizing antibody in 80 medical students of the University of Louisville. Six foamy virus researchers were also without these antibodies.

In the established taxonomy schemes for human viruses, the lower animal counterparts such as simian adenoviruses or picornaviruses are assigned serial serotype numbers separate from the human virus serial numbers. Therefore, the feline syncytial virus of Riggs et al. (9) and McKissick and Lamont (8) and the rabbit syncytium virus of Brown et al. (1) are not included in the simian foamy virus-numbering system, although they appear to be identical in many respects. Electron

microscopy of the latter virus (1) indicates that its morphology resembles that found by Clarke et al. (2) for foamy virus types 1, 2, and 3. Hackett et al. (4) have reported absence of neutralization of her strain of feline, syncytia-forming virus by the reference antisera to the 7 simian foamy virus types.

Preliminary experiments show lack of 5-iodo-deoxyuridine inhibition for virus types 1, 2, and 3, indicating that they are ribonucleic acid viruses; the same result was reported for type 6 and 7 by Hooks, et al. (Bacteriol. Proc., p. 179, 1969).

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