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Elimination of Persistent Simian Hemorrhagic Fever (SHF) Virus Infection in Patas Monkeys (42244)

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Abstract. Devastating epizootics of simian hemorrhagic fever (SHF) have been iatrogenically initiated in captive colonies of macaque monkeys by strains of SHF virus emanating from asymptomatic persistently infected patas monkeys. We have found that persistently infected patas monkeys can be cleared of their infection by superinfection with strains of SHF virus which cause acute infections in this species. All 20 persistently infected animals subjected to this procedure have been cleared of their infection within 3 months. These animals were shown to be virus free by the most sensitive *in vitro* and *in vivo* tests currently available and periodic tests of serum from these animals over several years have shown them to remain virus free. Superinfection has in some cases caused some adverse clinical symptoms (anorexia, lethargy, facial edema, dehydration, and mild subcutaneous hemorrhages), but with supportive care, no fatal infections have occurred. Thus, superinfection with acute strains of SHF virus is a highly effective method of eliminating persistent infection in patas monkeys.

Our previously reported serological and virus isolation studies have provided evidence that patas monkeys are a natural host of simian hemorrhagic fever (SHF) virus, a togavirus (1–3). Apparently, feral populations of patas monkeys are enzootically infected with SHF virus. We previously studied 216 animals born in their natural habit and found that 48.6% had antibody to SHF virus (1). This percentage may be somewhat high because we cannot unequivocally exclude that some of these infections did not occur after capture in holding facilities of importers or during shipment in groups to their destination.

SHF virus infections of patas monkeys are manifested as either acute or persistent infections. The type of infection which occurs apparently is determined by traits inherent to particular virus isolates (3). Recently, we described four isolates of SHF virus. Two of these isolates (LVR, P-180) produced acute infections in patas monkeys and the others (P-248, P-741) long-term asymptomatic persistent infections (3). Regardless of the SHF virus type, infections in patas monkeys usually are not fatal. Isolates which produce acute infections, however, have induced the more severe disease symptoms and the best humoral immune responses (3). Of the four isolates mentioned above, P-180 has consistently caused the most life-threatening disease (3).

Persistently infected patas monkeys have shown no clinical signs or symptoms of infection, even though they have been viremic for years (2). Furthermore, persistently infected mothers have given birth to and have reared babies which rarely have become infected (1). Babies do not appear to be immune to infection because of maternally acquired antibodies. Indeed, only low levels of specific antibodies have been detected in persistently infected animals (3). Newborns parenterally inoculated with homeotypic virus, however, were susceptible to infection. Therefore, most likely they were not infected *in utero* because virus did not cross their mother's placenta. The mechanisms by which babies are protected from *in utero* infection by their persistently infected mothers, however, have not been determined.

Iatrogenic spread of SHF virus from acute or persistently infected animals has been documented (2, 4). Poor animal care practices have been implicated in the near complete annihilation of colonies of captive macaque monkeys. Such practices as dispensing drugs, medicines, or nutritional supplements to animals from multidose vials with a common needle and syringe and tatooing animals without decontaminating the needle between each procedure have been suggested as likely means of virus transmission (2). An insect has not

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been identified as an intermediate host and vector in SHF virus transmission, but mechanical spread by biting insects cannot be excluded. Another possible means of spread is through open wounds caused by bites from infected animals. We believe that any mechanism by which SHF virus can be parenterally introduced into susceptible animals can be a potential means of initiating infection and spreading the disease.

Because of the difficulty in identifying persistently infected animals and the potential for accidental spread of SHF virus to the extremely sensitive macaque species, reliable methods to detect and eliminate persistent infections in patas monkeys have been sought. Previously we reported that cells of monocyte–macrophage lineage were the likely target of SHF virus in macaque species (5). From this finding, we developed a method employing *in vitro* cultured macrophages from rhesus monkeys by which chronic carriers of SHF virus can be detected (5). In this communication a method is described by which persistently infected patas monkeys can be cleared of infection. Clearance is effected by superinfection of these animals with heterotypic strains of SHF virus which cause acute infections.

Materials and Methods. *SHF virus isolates: Growth, purification, and infectivity assays.* The isolates of SHF virus used in this study were LVR 42-0/6941 (subsequently referred to as LVR), P-180, and P-248. LVR and P-180 produce acute infections in patas monkeys and P-248 a persistent infection. The origin and characteristics of these isolates have been described in detail in previous publications (3, 4, 6).

The methods used to grow, purify, and assay the infectivity of these isolates have also been described previously (1, 3, 5).

Antibody determinations. Titers of serum antibodies to specific isolates of SHF virus were determined by an enzyme-linked immunosorbent assay (ELISA) or by a virus neutralization test. The methods by which these tests were performed have been described elsewhere (3, 7).

Clearance studies: Age, sex, route of inoculation, and caging of animals. Of the 20 persistently infected patas monkeys involved in our clearance studies, 16 were adults (>3 years of age) and 4 were juveniles ranging in age from 7 months to 2 years. Eight adult animals were female and 8 were male. All 4 juvenile animals were also male. Twelve of the 20 animals studied acquired their persistent infections by natural means and 8 were experimentally infected. To clear persistent infections, all animals were inoculated intravenously with about 10^6 $TCID_{50}$s of an acute strain of SHF virus, i.e., either LVR or P-180. After persistently infected animals were superinfected with an acute strain of virus, their serum was monitored periodically for infectious virus by inoculation into *in vitro* cultured rhesus monkey PMAC. When several consecutive serum samples were found to be virus free by this procedure, a confirmatory test was performed by inoculating serum from the apparently cleared animals into macaque species which are highly susceptible to lethal infection by all known strains of SHF virus.

To minimize the chance of accidentally spreading SHF virus to macaque monkeys in our colony, all patas monkeys involved in clearance studies were housed in Horsfall isolator units that met the NIH size guidelines for patas monkeys.

Results. *Superinfection of persistently infected animals with acute strains of SHF virus.* Previously we reported that patas monkeys could be persistently infected with SHF virus after they had recovered from an acute infection and had high titers of antibody (3). These results are illustrated schematically by the first 4 monkeys, left to right, in the upper portion of Fig. 1. Since a persistent SHF virus infection could be established in antibody-positive animals that had previously experienced an acute infection, we were interested in the outcome of the opposite experiment, i.e., "Can persistently infected animals be superinfected with an acute strain of virus?" To initiate this experiment, patas monkeys Nos. 678 and 679 were inoculated with 10^4 $TCID_{50}$ of P-248 strain SHF virus. An infection of the persistent type was established in these animals (Table I). This point is supported by the long duration of the infection, by the fact that titers of infectious virus did not wane over this duration, by the low levels of specific antibody elicited by infection and by the unique ability of persistent strains of virus to lytically infect peri-

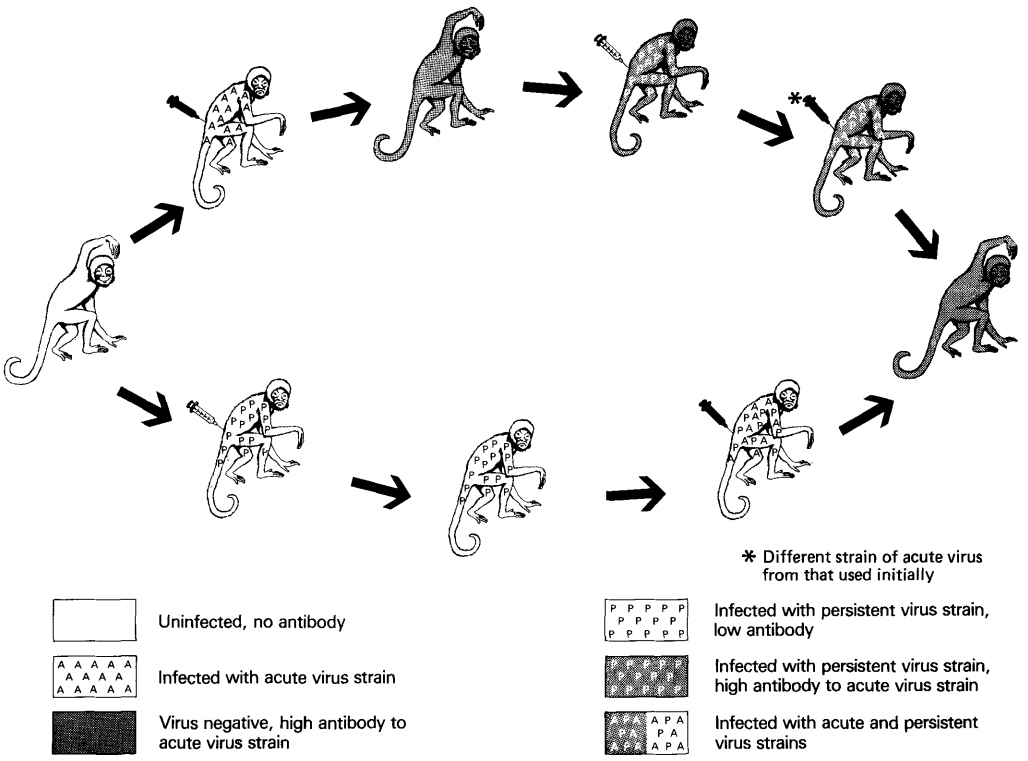

FIG. 1. Sequential infection of patas monkeys with acute and persistent strains of SHF virus and vice versa. The animals shown in the figure portray schematically the outcomes of these infections. See key above.

toneal macrophages from rhesus monkeys, but not from patas monkeys nor USU-104 cells (3).

After patas monkeys Nos. 678 and 679 had been persistently infected with P-248 virus for 147 days, they were inoculated with 10^6 $TCID_{50}$ of either LVR or P-180 (acute strains of SHF virus). No. 678 was inoculated with LVR and No. 679 with P-180. We previously reported that LVR had the capacity to lytically infect USU-104 cells and both patas and rhesus monkey PMAC, whereas the P-180 isolate only could lytically infect the two types of PMAC (3). Note that 1 day after superinfection, virus present in serum from No. 678 had the infectivity characteristics of LVR and virus present in serum from No. 679, those of P-180 (Table II). Infectious virus was detected for up to 14 days in the serum of No. 678 and for up to 42 days in serum of No. 679 (Table II). These results suggested that superinfection of persistently infected patas monkeys with acute strains of virus elicited a response which led to elimination of both the acute and persistent infections in these animals.

Eighteen additional persistently infected patas monkeys have similarly been superinfected with an acute strain of SHF virus. Persistent infection was eliminated in all 18 of these animals with a time course of elimination and antibody responses similar to those shown in Tables II and III for patas monkeys 678 and 679. By 90 days after superinfection, infectious virus was not detected in serum from any of these animals by *in vitro* tests in rhesus monkey PMAC or by *in vivo* tests in susceptible macaques. Once cleared of infection, periodic *in vitro* and *in vivo* tests of serum from several of these animals over a period of years has shown them to remain virus free. Thus, it appears that these animals truly have been cleared of persistent infection.

TABLE I. PERSISTENT INFECTION OF PATAS MONKEYS WITH STRAIN P-248 SHF VIRUS

Days after P-248 inoculation	Infectivity titer[a] Rhesus monkey PMAC[b]	Patas monkey PMAC	USU-104[c]
	A. Patas monkey No. 678		
0	<0.7	<0.7	<0.7
14	3.7	<0.7	<0.7
28	3.2	<0.7	<0.7
42	4.2	<0.7	<0.7
69	4.2	<0.7	<0.7
147	5.2	<0.7	<0.7
	B. Patas monkey No. 679		
0	<0.7	<0.7	<0.7
14	2.7	<0.7	<0.7
28	4.7	<0.7	<0.7
42	4.2	<0.7	<0.7
69	4.7	<0.7	<0.7
147	4.2	<0.7	<0.7

[a] $Log_{10}TCID_{50}$/ml or PFU/ml (USU-104).
[b] PMAC, peritoneal macrophages.
[c] USU-104, cell line from rhesus monkey embryonic kidney.

Antibody response in superinfected animals. The antibody responses of the superinfected animals were studied by ELISA and virus neutralization to determine whether induction of specific antibodies might have been the mechanism by which the persistent infection was eliminated. Titers of antibody in serial serum samples from the superinfected animals were determined by ELISA. The ELISA antigens used in these tests consisted of the strains of SHF virus to which these animals were exposed. As expected from results of our previous studies (3), only low titers of antibody to P-248 virus (persistent strain) were present in serum from these animals during the time course they were persistently infected (Table III, first 147 days). Titers of antibodies to viral antigens began to increase in sera from patas monkeys Nos. 678 and 679 7 days after they were superinfected and reached a maximum of 6250 around 28 days after they were superinfected (Table III). In both animals, the antibody elicited appeared to be primarily to the acute strain of virus (LVR or P-180), rather than the persistent strain (P-248). The slight rise in antibody titer (50 to 250) to the persistent strain of virus was probably the result of cross-reaction to epitopes shared by the acute and persistent strains of virus, since we previously showed them to be antigenically related, but not identical (3).

The same serial samples shown in Table III were also tested for their neutralizing activity to P-248, the persistent strain of virus. At no time point shown in Table III were significant titers of complement-dependent or independent neutralizing antibodies (<1:4 dilution of serum) detected in serum from patas monkeys Nos. 678 and 679 to P-248 virus. These results suggest that the probable mechanism of clearance was not via neutralizing antibodies.

Are animals cleared of persistent infection immune to reinfection? Patas monkeys Nos. 678 and 679 were reinoculated with 10^4

TABLE II. SUPERINFECTION OF PERSISTENTLY INFECTED PATAS MONKEYS WITH LVR OR P-180 STRAIN SHF VIRUS

Days after LVR inoculation	Infectivity titer[a] Rhesus monkey PMAC[b]	Patas monkey PMAC	USU-104[c]
A. Patas monkey No. 678; secondary inoculum–LVR			
0 (147)[d]	5.2	<0.7	<0.7
1 (148)	6.7	5.5	5.5×10^7
3 (150)	5.0	3.2	4.5×10^4
5 (152)	4.5	3.7	8.7×10^3
7 (154)	6.0	5.7	2.9×10^5
14 (161)	3.5	4.2	<0.7
21 (168)	<0.7	<0.7	<0.7
28 (175)	<0.7	<0.7	<0.7
35 (182)	<0.7	<0.7	<0.7
42 (189)	<0.7	<0.7	<0.7
B. Patas monkey No. 679; secondary inoculum—P-180			
0 (147)[d]	4.2	<0.7	<0.7
1 (148)	7.2	7.5	<0.7
3 (150)	5.5	7.5	<0.7
5 (152)	7.0	6.2	<0.7
7 (154)	6.0	5.7	<0.7
14 (161)	4.5	3.7	<0.7
21 (168)	5.5	4.0	<0.7
28 (175)	4.2	4.0	<0.7
35 (182)	<0.7	<0.7	<0.7
42 (189)	<0.7	0.7	<0.7
49 (196)	<0.7	<0.7	<0.7

[a] $Log_{10}TCID_{50}$/ml or PFU/ml (USU-104).
[b] PMAC, peritoneal macrophages.
[c] USU-104, cell line from rhesus monkey embryonic kidney.
[d] Numbers within parentheses are days after inoculation with P-248 SHF virus.

TABLE III. IgG ANTIBODY RESPONSES OF PERSISTENTLY INFECTED PATAS MONKEYS SUPERINFECTED WITH LVR OR P-180 STRAIN SHF VIRUS

Inoculum	Days after infection	ELISA antigen P-248	ELISA antigen LVR
	A. Patas monkey No. 678		
P-248 Strain	0	<10	<10
P-248 Strain	42	50[a]	50
P-248 Strain	147	50	50
LVR Strain	1 (148)[b]	50	10
LVR Strain	3 (150)	50	10
LVR Strain	5 (152)	50	10
LVR Strain	7 (154)	250	250
LVR Strain	14 (161)	250	1250
LVR Strain	21 (168)	250	1250
LVR Strain	28 (175)	250	6250
LVR Strain	42 (189)	250	6250
			P-180
	B. Patas monkey No. 679		
P-248 Strain	0	<10	<10
P-248 Strain	42	50[a]	50
P-248 Strain	147	50	50
P-180 Strain	1 (148)[b]	50	10
P-180 Strain	3 (150)	50	10
P-180 Strain	5 (152)	50	50
P-180 Strain	7 (154)	250	250
P-180 Strain	14 (161)	250	250
P-180 Strain	21 (168)	250	1250
P-180 Strain	28 (175)	250	6250
P-180 Strain	35 (182)	250	6250
P-180 Strain	42 (189)	250	6250
P-180 Strain	49 (196)	250	6250

[a] Reciprocal of the serum dilution yielding a positive ELISA result (≥ 0.2 A_{405} units above background).

[b] Numbers within parentheses are days after inoculation with P-248 SHF virus.

$TCID_{50}$ of homeotypic P-248 virus, 97 and 69 days, respectively, after they had cleared their infection. The purpose of reinoculating these animals was to determine whether persistent infections could be reestablished in them with P-248 virus, the homeotypic strain of virus, and thus evaluate their immunity to virus challenge. As shown in Table IV, infectious virus was only detected in the serum of these animals 1 and 3 days after reinoculation. By 6 days after reinoculation, serum samples from both animals were free of detectable virus. When these animals were again challenged with P-248 virus 8 months after they were cleared of persistent infection, similar results were also obtained (data not shown).

All of the serum samples listed in Table IV were tested for neutralizing antibodies to P-248 virus, but all were found to be negative (<1:4 dilution of serum). Thus, these results show that animals cleared of persistent infection with P-248 virus could be reinfected with homeotypic virus, but the infection was rapidly cleared by a mechanism(s) which has not been determined.

Discussion. The outcomes of sequential infection of patas monkeys with persistent and acute strains of SHF virus and vice versa are depicted schematically in Fig. 1. As portrayed in the lower scheme, pristine animals inoculated with persistent strains of virus (e.g., P-248, P-741) develop long-term, probably lifelong, asymptomatic chronic infections (3). Persistently infected patas monkeys cannot be visibly differentiated from normal healthy animals even though they are continuously viremic. Persistent infections of patas monkeys elicit only low levels of specific antibodies without virus neutralizing activity (3). Nevertheless, immune mechanisms probably participate in controlling persistent SHF virus infections of patas monkeys because persistently infected animals have been free of disease symptoms and the titers of infectious virus present in their serum have been relatively low and somewhat constant.

TABLE IV. PATAS MONKEYS WHICH HAVE BEEN CLEARED OF PERSISTENT INFECTION RESIST REESTABLISHMENT OF PERSISTENT INFECTION BY HOMEOTYPIC (P-248) VIRUS

Patas monkey No.	Days after reinoculation	Infectivity titer ($log_{10}TCID_{50}$/ml)
678	0 (97)[a]	<0.7
	1 (98)	5.2
	3 (100)	6.2
	6 (103)	<0.7
	10 (107)	<0.7
	15 (112)	<0.7
	18 (115)	<0.7
679	0 (69)	<0.7
	1 (70)	5.0
	3 (72)	5.2
	6 (75)	<0.7
	10 (79)	<0.7
	15 (84)	<0.7
	18 (87)	<0.7

[a] Numbers within parentheses are days after monkeys were cleared of previous SHF virus infections.

Although persistent strains of virus produce asymptomatic infections in patas monkeys, they produce highly lethal infections in macaque monkeys. Virus from persistently infected animals has been implicated in the iatrogenic initiation of several devastating epizootics of SHF in macaque colonies (2). Thus, simple reliable and inexpensive methods to detect and eliminate persistent infections in patas monkeys have practical value.

A problem which previously compounded the difficulty of detecting chronic carriers of SHF virus was the failure to identify cells which were susceptible to lytic infection by persistent strains of virus. An *in vitro* method for detection of persistently infected animals emanated from our identification of cells of monocyte–macrophage lineage as the target of lytic infection by persistent strains of virus (5). Most persistently infected animals can be identified by use of this *in vitro* technique. Occasionally, however, persistently infected animals have been missed by this technique. Apparently, the animals not detected by the *in vitro* procedure have very low titers of infectious virus. These animals, however, have been detected by inoculation of susceptible macaque species such as rhesus (*Macaca mulatta*) or cynomologous (*Macaca fascicularus*) monkeys with serum from the animals in question. Macaque inoculation still appears to be the most sensitive method available for detecting persistently infected animals, but it is very expensive and wasteful of animal resources.

In this communication, we have presented data showing that patas monkeys persistently infected with SHF virus can be cured of their infection by superinfection with an acute strain of SHF virus (e.g., LVR, P-180). The reliability of this method is emphasized by our success in curing chronic infections in all 20 animals subjected to this procedure. The clearance of persistent infection in these animals was verified both by *in vitro* and *in vivo* tests. Animals were cleared of persistent infection by 90 days after superinfection and have remained free of infection over the several years they have been studied. These results strongly suggest that our superinfection procedure has cleared persistent infections of patas monkeys.

Disease symptoms such as anorexia, lethargy, facial edema, dehydration, and mild subcutaneous hemorrhaging were seen in superinfected animals. The severity of the disease symptoms varied among superinfected animals. While some animals experienced fairly severe disease, most had only mild symptoms. Fatal infections, however, did not occur. The animals subjected to these procedures were housed in isolator cages and were given supportive care when needed. Superinfection of groups of animals housed in outdoor facilities to eliminate persistent infections has not been studied. Therefore, the safety of such a procedure has not been validated and must be approached with caution.

Previously we reported that persistent SHF virus infections could be established in patas monkeys which had recovered from acute infection and were antibody-positive and virus free (3). This point is illustrated diagrammatically in the upper pathway of Fig. 1. Secondary infection of persistently infected animals with the same acute strain of virus as that responsible for the initial infection reduced the titer of infectious virus, but did not eliminate persistent infection in the few animals studied. Nevertheless, superinfection of such animals with a different acute strain of virus did eliminate persistent infection. Therefore, knowledge of infection history can be a valuable adjunct in choosing the acute strain of virus to be used for clearance.

The mechanism by which persistent SHF virus infections of patas monkeys have been cleared by superinfection has not been elucidated. Significant titers of complement-dependent or independent neutralizing antibodies to persistent strains of SHF virus have not been detected in superinfected animals. However, significant titers of neutralizing antibodies do develop in patas monkeys infected with acute strains of SHF virus (3). Furthermore, our preliminary data suggest that neither complement-mediated nor antibody-dependent cellular cytotoxicity are the mechanisms by which persistent infections are cleared (M. Gravell, unpublished data). Clearance, however, appears to be mediated by an immune mechanism because on challenge with the homeotypic persistent strain of virus, about 3 and 8 months after the persistent infections had been eliminated, animals rapidly cleared these infections (see Table IV). This suggests that immunological memory cells were elicited in the initial clearing process. These results

imply that persistent strains of virus alone cannot initiate the steps leading to clearance, but can if assisted by acute virus strains. Our future work will be focused on understanding the mechanism of SHF virus persistence and clearance.

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