Simian Haemorrhagic Fever (SHF): New Virus Isolate from a Chronically Infected Patas Monkey

By MANETH GRAVELL, WILLIAM T. LONDON, MARCOS RODRIGUEZ, AMOS E. PALMER AND REBECCA S. HAMILTON

Infectious Diseases Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205, U.S.A.

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SUMMARY

A new strain of simian haemorrhagic fever (SHF) virus was isolated from chronically infected patas monkey no. 248 (P-248) in USU-104 cells. The P-248 isolate had the same size, morphology and cytoplasmic site of replication as the prototype LVR strain. However, the P-248 isolate caused a persistent infection without noticeable cytopathology in USU-104 cells rather than the strongly lytic infection produced by prototype LVR virus. The capacity of P-248 virus to produce a persistent, non-lytic infection of USU-104 cells was a very stable characteristic of the isolate. Extensive serial passage of this isolate through USU-104 cells (over 50 passages) and rhesus monkeys (six passages) failed to unmask virus with lytic properties for USU-104 cells. Culture medium from persistently infected cultures assayed in rhesus monkey peritoneal mononuclear phagocytes, where measurable cytopathology occurs, was found to contain about $10^5$ to $10^6$ TCID$_{50}$/ml of cell-free P-248 virus. Immunolabelling techniques showed only a low percentage of infected cells in persistently infected cultures. The mechanism of persistence of the P-248 isolate in USU-104 cells has not been determined but evidence suggests it does not involve interferon or defective interfering particles.

INTRODUCTION

Simian haemorrhagic fever (SHF) is a febrile, rapidly fatal haemorrhagic disease of monkeys of genus Macaca, caused by a member of the Togaviridae family (Trousdale et al. 1975), tentatively subclassified as a pestivirus (Melnick, 1976). Several severe epizootics of SHF, involving captive colonies of monkeys, have been reported (Palmer et al. 1968; Myers et al. 1972; London, 1977; D. Dalgard, personal communication). In these epizootics, infection of various macaque species was nearly 100% fatal while monkeys of other genera and exposed humans were unaffected. Common laboratory animals, e.g. mice, rats, hamsters and guinea-pigs also were not found to be susceptible (Tauraso et al. 1968). These facts illustrate the extreme lethality and selective virulence of SHF virus for macaques.

The prototype strain of SHF virus (LVR 24-/M6941) was isolated from a stumptailed macaque (Macaca arctoides) involved in the Bethesda 1964 epizootic (Tauraso et al. 1968). This isolate, called LVR for brevity, produced typical haemorrhagic disease when inoculated into macaque monkeys and lytically infected a rhesus monkey kidney cell line.
yielding high titres of infectious virus. This cell line was formerly referred to as MA-104, but the currently preferred designation is USU-104 (Myers et al. 1972; M. M. Vincent, personal communication).

A number of patas monkeys (Erythrocebus patas) have been found to carry SHF virus for long periods, perhaps for life, without exhibiting symptoms of infection (London, 1977). Serum taken, apparently at any time, from these chronically infected animals will produce typical SHF disease if inoculated into macaques, suggesting that chronically infected animals are continuously viraemic (London, 1977). Unlike the prototype LVR strain, virus from chronically infected animals did not produce lytic infection of USU-104 cells, nor have methods to grow or titre this virus in vitro been reported. Several epizootics of SHF have also been reported in which a virus was not isolated in spite of serious attempts by qualified virologists (Myers et al. 1972).

Chronically infected patas monkeys pose a serious danger to macaque colonies because of the duration and asymptomatic nature of their infection. In fact, epizootics have been traced to the accidental infection of macaque monkeys with virus from patas monkeys (London, 1977). The extreme lethality of SHF virus for macaque monkeys, the lack of an in vitro test to detect chronic virus carriers and the paucity of information concerning the epidemiology, virology/immunology and pathogenesis of SHF prompted these studies. In this communication, we report the isolation and partial characterization of a strain of SHF virus from a chronically infected patas monkey.

**METHODS**

*Virus isolation from mononuclear leucocytes of a chronically infected patas monkey.* P-248 was received from the NCI colony where it was shown to be a chronic carrier of SHF virus on May 8, 1974. Peripheral blood mononuclear leucocytes (PBML) were isolated from heparinized blood withdrawn from P-248 by the Ficoll-Hypaque procedure of Boyum (1968). Heparinized blood was diluted 1:2 with Hanks' balanced salt solution (HBSS) and 10 ml of diluted blood layered on to a 3 ml cushion of Ficoll-Hypaque solution, specific gravity 1.08 (Microbiological Associates, Bethesda, Md., U.S.A.). Centrifugation was at 350 g for 30 min at room temperature. Mononuclear leucocytes were harvested and washed three times with HBSS. Between $10^6$ and $2 \times 10^6$ mononuclear leucocytes were obtained per ml of blood.

To isolate virus from mononuclear leucocytes of P-248, $5 \times 10^6$ mononuclear leucocytes were co-cultivated with $5 \times 10^5$ USU-104 cells in 25 cm² plastic culture flasks (Costar, Cambridge, Mass., U.S.A.). Antiserum to patas monkey syncytium-forming virus was included in the culture medium (Eagle's minimum essential medium with 10% foetal calf serum; Microbiological Associates) and 50 mg of gentamicin/ml (EMEM-10). Incubation was at 37°C in a humidified atmosphere of 5% CO₂ in air.

*Virus isolation from lymphoid tissues of an acutely infected rhesus monkey.* Mesenteric lymph node, inguinal lymph node, thymus and spleen were surgically removed aseptically from a terminally ill rhesus monkey (no. A862). These tissues were finely minced and each was co-cultured with USU-104 cells as described above. The virus producing the illness in rhesus monkey no. A862 was the sixth serial passage of P-248 virus in rhesus monkeys. This experiment was an attempt to unmask SHF virus from P-248 infections with lytic properties for USU-104 cells.

*Immunolabelling procedure.* Infected and control monolayers of USU-104 cells were grown to confluency on glass coverslips (12.5 mm), fixed in situ with 1% p-formaldehyde in 0.01 M-cacodylate buffer for 10 min, washed with phosphate-buffered saline (PBS), pH 7.2, overlaid with PBS, slowly frozen and stored at $-70°C$ until immunolabelled.
Diluted (1:20) immune or control serum was incubated with fixed cells on coverslips for 1 h at room temperature. Serum was removed from cultures and the cultures rocked while washing three times with PBS, 20 min/wash. Protein A-peroxidase was incubated with cultures for 1 h, the conjugate was removed and cultures washed three times as above. Infected cells were cytochemically stained by incubating cultures for 10 min at pH 7.6 with diaminobenzidine-peroxide substrate (Graham & Karnovsky, 1966).

**Microscopy.** Cultures infected with either lytic (LVR) or persistent (P-248) SHF virus were immunolabelled and cytochemically stained as described above. Cultures were examined with a light microscope and areas with stained cells photographed. Areas showing stained cells were processed for electron microscopy by standard methods. Thin sections were cut tangentially and sagittally to the monolayer with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Philips 201 electron microscope.

**Interferon assay.** Interferon assays were performed in 96 well, tissue culture grade, microtitre plates (Linbro Scientific, Hamden, Conn., U.S.A.). Plates were seeded at 20000 cells/well with low passages (8 through 12) of Flow 7000, human foreskin fibroblasts (Flow Laboratories, McLean, Va., U.S.A.); culture medium was EMEM-10. Confluent 1 to 2 day-old cultures of Flow 7000 cells were treated overnight with serial dilutions of samples being tested for interferon activity. The cultures were then washed twice with culture medium and each culture well was challenged with approx. 10⁴ TCID₅₀ of the Indiana strain of vesicular stomatitis virus (VSV). Tests were read for interferon activity when VSV-challenged controls (cultures not treated with interferon) showed total cytopathology.

**Replication of LVR virus or VSV in persistently infected cells.** A mechanism proposed to explain persistent infection of cell cultures is the generation of defective interfering particles. Using this concept, work was done to determine whether replication of LVR strain SHF virus or VSV was restricted in USU-104 cells persistently infected with P-248 virus.

Normal or persistently infected USU-104 cells (7th or 28th subculture after persistent infection with P-248 virus) were grown to confluency in 25 cm² plastic flasks (CoStar, Cambridge, Mass., U.S.A.). At confluency, the normal or persistently infected cultures each contained about 3 × 10⁶ cells. The input m.o.i. for these cultures was 1 for LVR virus and VSV. After 1 h at 37 °C for virus adsorption, cultures were washed three times with HBSS (5 ml/wash) and 5 ml of EMEM-10 was added to each culture. Cultures were incubated at 37 °C in an atmosphere of 5% CO₂ in air. Samples were taken by rapidly freezing individual cultures in dry ice at 2 h or 26.5 h p.i. Yields of LVR virus or VSV were determined by plaque assay in USU-104 cells (Gravell et al. 1980).

**RESULTS**

**Isolation of SHF virus from a persistently infected patas monkey**

The prototype strain of SHF virus (LVR) has been shown to produce typical haemorrhagic disease in macaques and to lytically infect USU-104 cells, totally destroying cell monolayers within 2 days p.i. Although sera from persistently infected patas monkeys contained virus which produced typical SHF when inoculated into macaques, virus could not be isolated from these sera in USU-104 or other cell lines. This implied that the virus present in persistently infected patas monkeys differed from the prototype LVR strain.

To test the possibility that putative antibody present in sera from chronically infected patas monkeys might be responsible for these unsuccessful *in vitro* isolation attempts, co-cultivation studies were initiated. Transmission studies in macaques showed persistently
infected patas monkeys to be constantly viraemic. Therefore, peripheral blood mononuclear leucocytes (PBML) were suspected to be target cells which maintained the infection.

PBML from P-248 were separated from heparinized blood by centrifugation on a cushion of Ficoll–Hypaque solution. Washed PBML were co-cultured with USU-104 in an attempt to isolate SHF virus. The only cytopathology seen in these co-cultures was from patas monkey foamy virus, since shown to be a new serotype of the simian foamy virus group (Kiefer, 1980). To inhibit replication of this foamy virus, antisera with neutralizing activity was included in the culture medium. No cytopathology developed when P-248 PBML were co-cultured with USU-104 cells or other cell lines from monkeys (Vero, CV-1) in medium containing patas monkey foamy virus antisera. In addition, cytopathology also did not develop when cells of these lines were co-cultured with finely minced lymphoid tissues (lymph node, spleen and thymus) from rhesus monkeys fatally infected with P-248 virus. Transmission studies in macaques showed these lymphoid tissues to contain infectious SHF virus.

To determine whether SHF virus from P-248 might be replicating in USU-104 cells without producing marked cytopathology, a rhesus monkey was inoculated with 1 ml of tissue culture medium from a co-culture of P-248 PBML and USU-104 cells. This co-culture had been passaged five times in vitro, at approximately weekly intervals, using a 1:5 split ratio. Medium from this culture contained virus which produced SHF disease in the inoculated rhesus monkey. To eliminate the possibility that the virus causing SHF in this rhesus monkey was carried over from the initial cell inoculum rather than from replication in vitro, additional cell passages were made. Inoculation of a rhesus monkey with fluid from the 28th, fivefold subculture (approx. 6 months of in vitro cultivation, >10^20 dilution of the original material) still caused SHF, providing convincing evidence for replication and persistent infection of USU-104 cells by P-248 SHF virus.

**Studies on persistently infected cells**

The percentage of infected cells in persistently infected USU-104 cultures was determined by use of a protein A–peroxidase immunoassay. USU-104 cells persistently infected with the P-248 isolate or lytically infected with prototype LVR virus were treated with immune or preimmune patas monkey serum, washed, labelled with protein A–peroxidase and stained with diaminobenzidine-peroxide substrate. Uninfected control cells were processed similarly. Low magnification photomicrographs of lytically or persistently infected cells (22nd in vitro passage) are shown in Fig. 1 (a and b respectively). Note that most cells in the lytically infected culture (20 h p.i.) were darkly stained (evidence of infection) and virus-induced cytopathology was already evident in the culture (Fig. 1a). In contrast, only a few darkly stained cells were present in the persistently infected culture and cytopathology was not evident (Fig. 1b). Tests of persistently infected cells from other passages gave similar results. These results suggest that only a low percentage of cells in the persistently infected USU-104 cultures were infected at any particular time. Preliminary cloning experiments support this conclusion.

When the persistently infected cultures were established and during their early passage history, the only method available to monitor virus production by these cultures was to inoculate susceptible macaque monkeys and observe for fatal infection. Because of the high cost and shortage of these animals, it was not possible to determine titres of virus produced by the persistently infected cells. Recently a method employing mononuclear phagocytes was developed by which virus from persistently infected cultures or animals can be titrated (M. Gravell et al., unpublished data). By use of this method, we have found that about 10^5 to 10^6 TCID_{50}/ml of non-cell-associated virus are present in culture
Fig. 1. (a) USU-104 cells lytically infected with the prototype LVR strain of SHF virus, 20 h p.i. at 1 p.f.u./cell. Infected cells were labelled and stained by the immunoperoxidase technique using diaminobenzidine as substrate. (b) USU-104 cells persistently infected with the P-248 isolate of SHF virus, 22 passages after infection. The dark cells in the photograph are infected. They were labelled by the immunoperoxidase technique and stained by reaction with diaminobenzidine substrate. (c) Electron micrograph of virus particles with togavirus morphology inside a vacuole of an immunoperoxidase-labelled, diaminobenzidine-stained cell from a USU-104 culture persistently infected with the P-248 isolate of SHF virus. These virus particles are morphologically identical to virions seen in cells lytically infected with prototype LVR virus (see inset).
medium of persistently infected USU-104 cultures. We have also found that persistent infection of USU-104 cells can be initiated by direct inoculation of virus from persistently infected patas monkeys and does not require co-cultivation techniques.

**Ultrastructure of persistent virus**

The morphology and ultrastructural aspects of replication of the P-248 isolate were compared with those of prototype LVR virus. Because only a low percentage of the cells in persistently infected cultures contained virus, the chance of sectioning infected cells by random cutting was low. To increase this probability, infected cells were immunolabelled and stained with diaminobenzidine. Areas showing stained cells were processed for electron microscopy, thin-sectioned and stained. By this technique, the size and morphology of the P-248 isolate were found to be identical to LVR. Furthermore, both LVR and P-248 virus bud into cytoplasmic vacuoles rather than from the plasma membrane, a characteristic of the genus pestivirus of the *Togaviridae* family (Fig. 1c).

**Stability of the non-lytic nature of the P-248 isolate for USU-104 cells**

A rhesus monkey was inoculated with 1 ml of serum containing about $10^4$ TCID$_{50}$ of virus from persistently infected P-248. When the inoculated rhesus monkey developed advanced SHF disease, serum from the moribund animal was injected into another rhesus monkey. Six serial passages of P-248 virus through rhesus monkeys were made using this procedure. The objective of passage of P-248 virus through rhesus monkeys was to determine whether a virus with lytic properties for USU-104 cells could be selected by this procedure and ultimately isolated.

Mesenteric and inguinal lymph nodes, thymus and spleen were surgically removed aseptically from the sixth consecutive rhesus monkey (no. A862) receiving P-248 virus. These tissues were finely minced and each was co-cultured with USU-104 cells. Only SHF virus which was non-lytic for USU-104 cells was isolated from all of the above tissues suggesting that the non-lytic trait is a stable characteristic of P-248 virus. Further support for this conclusion comes from the fact that to date, P-248 virus-infected USU-104 cells have been subcultured over 55 times *in vitro* (fivefold split ratio) and virus produced was still non-lytic for these cells.

**Mechanism of persistence of P-248 virus in USU-104 cells: are defective interfering particles or interferon involved?**

Other studies have shown that interferon or defective interfering particles are factors frequently involved in establishing and maintaining persistent virus infections. To determine whether either of these factors might account for the persistence of strain P-248 SHF virus in USU-104 cells, culture medium from persistently infected cultures was tested for interferon and evidence for defective interfering particles was sought by challenging persistently infected cultures with either a closely related virus (LVR strain of SHF virus) or an unrelated virus (VSV, a rhabdovirus) and determining the impact of persistent infection on yields of challenge viruses. Interferon was never detected in culture medium from various passages of persistently infected cells nor in medium taken at various times within particular passages (data not shown). Furthermore, no evidence was obtained that defective interfering particles were the cause of persistence of P-248 virus in USU-104 cultures. Normal cytopathology developed and no restriction in replication of LVR virus or VSV was noted in persistently infected cultures. Yields of virus from normal and persistently infected cultures were essentially the same (Table 1). Thus, the mechanism of persistence of P-248 virus in USU-104 remains to be determined.
Persistent strain of SHF virus

Table 1. Does persistent infection of USU-104 cells with P-248 SHF virus interfere with replication of LVR virus or VSV?

<table>
<thead>
<tr>
<th>USU-104 cells</th>
<th>Time p.i. (h)</th>
<th>Infectivity (p.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2</td>
<td>$4 \times 10^9$</td>
</tr>
<tr>
<td></td>
<td>26.5</td>
<td>$1.7 \times 10^9$</td>
</tr>
<tr>
<td>Persistently infected, P-7*</td>
<td>26.5</td>
<td>$1.6 \times 10^9$</td>
</tr>
<tr>
<td>Persistently infected, P-28*</td>
<td>26.5</td>
<td>$1.3 \times 10^8$</td>
</tr>
</tbody>
</table>

* P-7 and P-28 refer to 7th and 28th subcultures, respectively, after persistent infection with P-248 virus.

**DISCUSSION**

The LVR and P-248 isolates of SHF virus have characteristics which suggest they are related but not identical. Both isolates produced a clinically similar fatal hemorrhagic disease in macaque monkeys and by electron microscopy had the same size, morphology and cytoplasmic site and mode of replication. The isolates, however, were found to differ antigenically. Antisera with neutralizing activity to LVR virus did not neutralize P-248 virus. Furthermore, reciprocal enzyme immunoassays showed antisera to react with homologous virus to a higher titre than with heterologous virus (M. Gravell, unpublished data). In addition, different types of infection were produced by these isolates in USU-104 cells. Whereas the LVR isolate produced a rapid, markedly lytic infection, the P-248 isolate produced a persistent infection with only minimal cytopathology. USU-104 cells persistently infected with P-248 virus have been passaged over 50 times in vitro using a 1:5 split ratio without selecting virus with lytic properties for these cells. In addition, P-248 virus has been passaged serially through six rhesus monkeys causing a fatal infection in each without selecting virus with lytic properties for USU-104 cells. Thus, the non-lytic, persistent nature of the P-248 isolate is a very stable trait.

Infection of patas monkeys with LVR or P-248 virus resulted in infections paralleling those found in USU-104 cells. An asymptomatic, long-term, persistent infection was produced in all six patas monkeys inoculated with P-248 virus. In contrast, both patas monkeys inoculated with LVR virus developed an acute infection with only mild clinical manifestations. LVR virus was no longer detectable in these animals between 1 and 2 months p.i. These results, although preliminary, strongly suggest that the major factor determining whether a patas monkey will become chronically infected with SHF virus is the genotype of the infecting virus strain rather than genetic differences between infected animals. They further indicate that the LVR and P-248 isolates are distinct, naturally occurring strains of SHF virus.

The mechanism of persistence of P-248 virus in USU-104 cells or in chronically infected patas monkeys is unknown. Our preliminary experiments did not implicate interferon or defective interfering particles as the cause of persistence; however, additional work will be required to rule out these factors completely. Future studies will be directed towards understanding whether the P-248 isolate has unique features which allow it to persist in USU-104 cells and patas monkeys.

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REFERENCES


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