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Simian Hemorrhagic Fever Virus: A New Togavirus (39111)

M. D. TROUSDALE, D. W. TRENT[1], AND A. SHELOKOV

Department of Microbiology, The University of Texas Health Science Center, San Antonio, Texas 78284

Several explosive epizootics of simian hemorrhagic fever (SHF) affecting monkeys of three Macaca species, rhesus (*M. mulatta*), cynomolgus (*M. irus*), and stump-tailed macaque (*M. speciosa*), have occurred during the past decade in Soviet, American, and British vivaria. The two initial outbreaks were recognized a few weeks apart in 1964 at the Institute of Experimental Pathology and Therapy, Sukhumi, U.S.S.R. (1), and at the quarantine colony of the National Institutes of Health (NIH), Bethesda, Maryland (2), among monkeys supplied from India to both institutions by the same shipper. During the late sixties other epizootics were reported, one from the National Center for Primate Biology, Davis, California, in 1967, and several from the primate colony in Sussex, U. K. (1966–69) (3). The most recent epizootic, involving over 200 monkeys, occurred in 1972 at the NIH (4).

The clinical and pathological features of each SHF outbreak have been similar, and the SHF virus strains isolated from the sick monkeys were immunologically related (1–7). The prototype Bethesda virus was shown to be enveloped, pH-sensitive, relatively heat-stable, and 40–45 nm in diameter; the results of metabolic inhibition studies have suggested that the viral genome was RNA (3, 5, 7).

Although it has been noted that the SHF virus resembles the arboviruses of group B in many of its properties (7), the virus has not yet been classified. The purpose of this study was to define further the biophysical and biochemical properties ot the prototype strain of SHF virus.

Materials and methods. The prototype LVR 42-0/M6941 strain of SHF virus (5, 6), received from Dr. N. M. Tauraso, was propagated in MA-104 embryonic rhesus monkey kidney cells originally obtained from Microbiological Associates, Inc. The cells were maintained in Medium 199 containing neomycin (50 μg/ml) and 10% newborn calf serum, and they were inoculated with the virus at a multiplicity of infection 0.1, the maximum allowed by the seed virus titer. For experiments involving radioisotopes, three media were used: for nucleic acid and phospholipid studies, a phosphate-free medium with ^{32}P (2.5 μCi/ml); and for structural protein studies either of two amino acid-free media, one containing ^{3}H-leucine and ^{3}H-valine (total 5 μCi/ml), and the other containing ^{14}C-protein hydrolysate (5 μCi/ml) and ^{3}H-glucosamine (0.5 μCi/ml). The radioisotope-containing media were added after virus inoculation and the cultures were incubated at 37°. Virus was harvested 48–72 hr after infection, purified, and concentrated as previously described for arboviruses (8). Virus preparations were then fixed in 1% osmic acid vapor for 2–5 min, negatively stained with uranyl acetate, and examined by electron microscopy (9). A carbon replica calibration standard from Ernest Fullam Company was used to determine instrumental magnification.

For protein analysis by polyacrylamide gel electrophoresis (PAGE), radioactively labeled purified virions were solubilized by heating at 100° for 1 min with equal volumes of 1% sodium dodecyl sulfate (SDS), and 1% 2-mercaptoethanol. The PAGE analysis was done in 10% gels at 10 mA per gel (6 mm × 175 mm) (10). Nucleic acids were extracted with phenol-SDS from purified SHF virus and from Saint Louis encephalitis (SLE) virus, the latter being used as a control. Each extracted nucleic acid was precipitated with two volumes of 95% ethanol at −20° for 18 hr, collected by centrifugation, and the pellet was dissolved in a gradient buffer (0.1 *M* NaCl, 0.001 *M* Tris, pH 7.5, and 0.0001 *M* EDTA). The nucleic acids were electrophoresed in 2.4% acrylamide

[1] Current address: Vector-Borne Diseases Division, Center for Disease Control, Fort Collins, Colorado 80522.

and 0.6% agarose gels at 5 mA per gel (11). The gels were sliced into 1 mm segments and incubated overnight at 37° in capped vials containing hydrogen peroxide. After the addition of toluene-Triton x-100 counting fluid, the radioactivity was measured in a Beckman LS-230 liquid scintillation system and the data, recorded on paper tapes, were analyzed by an IBM Model 1130 computer.

Phospholipids were extracted with chloroform–methanol (2:1) from labeled purified SHF virus and from ^{32}P-labeled virus-infected and noninfected MA-104 cell cultures (12). Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on activated precoated, 0.25 mm, silica gel plates (EM Laboratories, Inc., Darmstadt, Germany), using chloroform–methanol–water (65:25:4) and butanol–acetic acid–water (60:20:20) (13). The ^{2}P-phospholipids were visualized by exposing the TLC plate to X-ray sheet film and comparing the exposure patterns to known reference spots that were identified by iodine vapor (14).

Results and discussion. SHF virus was sedimented to equilibrium in a potassium tartrate gradient. The peaks of radioactivity and optical density readings coincided and the peak fraction containing the labeled virus had an approximate density of 1.18 g/cm^3. Ninety-two particles in this fraction were measured, and their outer diameters varied from 40 to 100 nm, averaging 48 nm, with 93% measuring between 45 and 50 nm (Fig. 1). Most of the virions were spherical and possessed tight-fitting envelopes that masked the ultrastructure of the internal nucleocapsids (Fig. 1A); some tubular structures, 25 nm in diameter, were occasionally seen. The nucleocapsids of intact particles had an average diameter of 25 nm (Fig. 1B). These

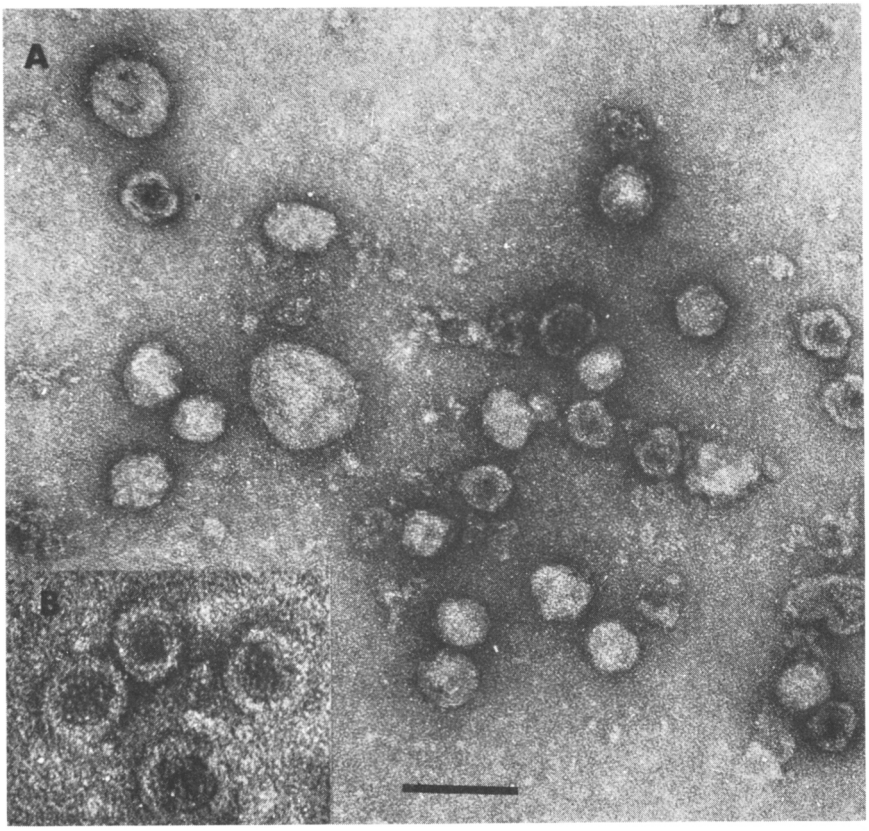

FIG. 1. A. Purified SHF virus particles negatively stained with unbuffered uranyl acetate. Inset B. Nucleocapsids outlined by uranyl acetate which has penetrated the envelopes. Bar is equal to 100 nm.

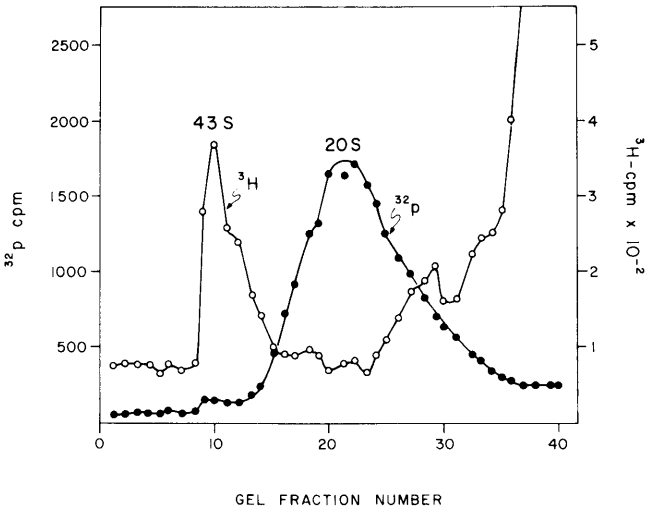

FIG. 2. Polyacrylamide gel coelectrophoresis of ^{32}P-labeled nucleic acid from SHF virus and ^{3}H-RNA from purified Saint Louis encephalitis virus. Electrophoresis was from left to right. Symbols: ● SHF nucleic acid; ○ SLE RNA.

measurements confirmed the findings of other investigators who, using thin-section electron microscopy, reported SHF virus particles in infected MA-104 cell cultures to be 40–45 nm in diameter (7).

Results of coelectrophoresis of ^{32}P-labeled nucleic acid extracted from purified SHF virions and ^{3}H-uridine-labeled RNA from SLE virions are shown in Fig. 2. The phenol-SDS extraction procedure that we usually employ does not disrupt intact RNA genomes. Hence, the broad peak of ^{32}P activity and the small S value (20S) for the SHF virus genome suggests that the genome may be segmented (15–30S), unless it is unusually fragile. This supposition is supported by the fact that the extract was noninfective, at least as judged by attempts at assaying infectious RNA in MA-104 cell cultures by two different methods (15, 16).

To determine the effect of enzyme digestion on the SHF virus genome, equal volumes of RNAse (25 μg/ml) or DNAse (50 μg/ml) were mixed with the ^{32}P-labeled nucleic acid from SHF virus and incubated at room temperature for 30 min; 91% of the nucleic acid was digested to nonacid precipitable fragments by RNAse, while the DNAse treatment decreased the total precipitable radioactivity of the SHF virus nucleic acid by 12% (probably owing to RNAse contamination of the commercial DNAse preparation). These results support the findings of others who, because BUDR did not inhibit SHF virus growth, had suggested that the SHF virus genome is RNA (5). Furthermore, our enzymatic studies indicated that the genome is single-stranded RNA.

Electropherograms of purified SHF virus doubly labeled with ^{14}C-amino acids and ^{3}H-glucosamine showed four peaks of radioactivity which we designated structural proteins SP-1 through SP-4; SP-1 and SP-4 were identified as glycoproteins by coelectrophoresis (Fig. 3). Most of the ^{3}H-glucosamine-labeled protein migrated in gel fractions that contained SP-1; much less ^{3}H-glucosamine-labeled material was found in fractions containing SP-4. Phospholipid content of purified SHF virions, in comparison with infected and noninfected MA-104 cells, was repeatedly determined, and a representative set of values is shown in Table I. The high percentage of phosphatidylcholine in the virions is notable. This finding, suggesting that the viral envelope originated in the internal membranes of the host cell (17), agrees with the electron microscopic findings of others (7).

Using biophysical and biochemical techniques, we have confirmed the earlier pre-

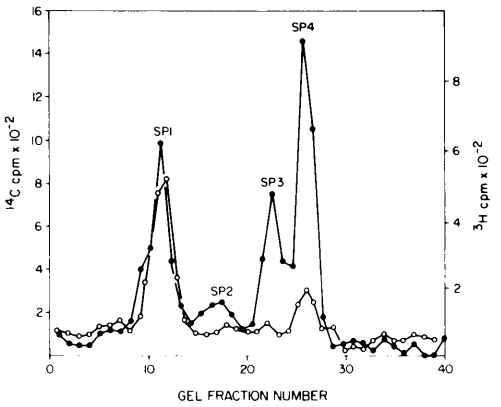

FIG. 3. Polyacrylamide gel electrophoresis of doubly labeled SHF virus structural proteins. Electrophoresis was from left to right. Symbols: ● ^{14}C-amino acids; ○ ^{3}H-glucosamine.

TABLE I. PERCENT OF TOTAL ^{32}P-PHOSPHOLIPIDS IN PURIFIED SHF VIRUS, INFECTED MA-104 AND NONINFECTED MA-104 CELLS

Phospholipid	Purified SHF virus (%)	SHF virus-infected MA-104 cells (%)	Noninfected MA-104 cells (%)
Phosphatidylcholine	65.0	51.9	45.5
Phosphatidylethanolamine	24.7	25.9	26.8
Phosphatidylinositol	1.0	2.5	2.2
Phosphatidylserine	—[a]	15.5	17.2
Lysophosphatidylcholine	1.9	1.8	0.2
Lysophosphatidylethanolamine	—[a]	0.4	0.5
Sphingomyelin	1.1	0.8	5.2
Other ^{32}P activity (not identified)	6.3	0.12	2.4

[a] Phospholipid not observed.

liminary characterization of SHF virus (3, 5, 7), and we have been able to explore further the nature of the viral genome, to define the viral structural proteins, and to determine the phospholipid content of the virions. The relevant results of the studies by us and by others can be arranged in the order of virus classification criteria promulgated by the International Committee on Nomenclature of Viruses (18) as follows: (1) The virus nucleic acid core is RNA; (2) the virus capsid symmetry is probably cubic; (3) the virion is enveloped; (4) the site of capsid assembly is in the cytoplasm; (5) the site of nucleocapsid envelopment is at the intracytoplasmic membranes; (6) the virion is sensitive to ether treatment; and (7) the diameter of the virion is from 40–50 nm. (The molecular weight of the togavirus nucleic acid is listed as 3×10^6 (18); this determination was not performed by us.) This description would place SHF virus into the newly proposed Togavirus family. Furthermore, two of these properties (small size and budding from an internal membrane) suggest that SHF virus may belong to the Flavivirus group.

Summary. Using the prototype strain of SHF virus, we have confirmed the nature of the genome (RNA), the presence of an envelope (derived from an internal membrane), the virion size (45–50 nm), and probable cubic symmetry. We have also described four viral structural proteins and determined the phospholipid content of the virions. The known properties of SHF virus suggest that it should be classified in the Togavirus family, and possibly in the Flavivirus group.

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