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**HANTAAAN VIRUS, AETIOLOGICAL AGENT OF  
KOREAN HAEMORRHAGIC FEVER, HAS  
BUNYAVIRIDAE-LIKE MORPHOLOGY,**

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**Summary** Spherical to oval particles with a unit membrane and subunit surface structure were demonstrated by negative-contrast staining of supernatant fluids of A-549 cell cultures infected with strain 76-118 of Hantaan virus. The particles had an average diameter of about 95 nm, with a range of 80 to 110 nm. Similar particles were isolated by buoyant density fractionation in sucrose gradients. In four separate experiments, infectivity co-sedimented with 95 nm particles at buoyant densities from 1.15 to 1.18 g/ml. Immunoaggregation of the virions was specifically produced by antisera obtained after Hantaan virus infection of man and rabbit. The known physicochemical and morphological properties of these particles are compatible with those generally reported for the Bunyaviridae family of viruses.

**Introduction**

ISOLATION of the elusive aetiological agent of Korean haemorrhagic fever (KHF) was first reported in 1978.<sup>1</sup> Partial characterisation of this agent, named Hantaan virus, led us to postulate that it is a medium-sized, enveloped, acid-sensitive, RNA virus.<sup>2,3</sup> In 1979, we succeeded in cultivating

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a single virus isolate, designated 76-118, in A-549 cell culture, a continuous cell line derived from a human lung carcinoma.<sup>2,4,5</sup> Infection of these cells was non-cytolytic, persistent, and best detected by immunofluorescence (IF) which revealed viral antigen confined to the cytoplasm in a discrete granular pattern.

Despite virtually complete infection of A-549 cells as indicated by IF, infectivity of virus harvests in supernatants rarely exceeded  $10^5$  infectious units/ml. Electron microscopy of sectioned cell pellets as well as negative-contrast preparations from supernatants demonstrated only occasional virus-like particles of approximately 95 nm diameter with poorly resolved substructure. Particles were suspiciously Bunyaviridae-like, but IF tests with ascitic fluids immune to many members of this large virus family were negative against Hantaan virus-infected cells.

A recent report<sup>6</sup> describing orbivirus-like particles in three strains of Hantaan virus, including strain 76-118, was at variance with some of the previously measured virus properties. This led us to use buoyant density centrifugation to re-examine Hantaan virus.

### **Materials and Methods**

#### *Virus and Antisera*

All work was done with virus pools of strain 76-118 in the 10th to 14th passages in A-549 cells. The origin and passage history of this virus have been described.<sup>1,2</sup>

Two antisera were used for immunofluorescent infectivity determinations and for immune electron microscopy (IEM). These were a rabbit antiserum to the heterologous Lee strain of Hantaan virus<sup>7</sup> or a human serum, 7000047, from an adult male convalescing from severe and clinically classic KHF. Rabbit and human antisera had IF titres of 1:32 000 and 1:2048, respectively, against 76-118. Antibodies to reovirus antigens types 1, 2, and 3 were not detected by IF at 1:4 dilutions of these sera.

#### *Cell Cultures and Virus Propagation*

A-549 cells were obtained from the American Type Culture Collection (CCL-185). The complete medium (CM) used for growth and maintenance of cells was Eagle's minimal essential medium (E-MEM) supplemented with 5% heat-inactivated fetal calf serum, 50 µg/ml gentamicin sulphate and 30 µg/ml 'Tylocine' (Gibco, Grand Island, New York). We found that culture fluids obtained 6 days after inoculation with 76-118 virus could be concentrated approximately 100-fold by centrifugation at 5000 g for 30 min followed by resuspension of pellets in an appropriately reduced volume. This concentrated material produced inocula

containing infectivity to cell ratios of about 2 in  $150 \text{ cm}^2$  (surface) flasks. Supernatants harvested daily from day 7 up to and including day 10 after infection were pooled and passed through a filter with a limiting pore diameter of  $0.8 \text{ }\mu\text{m}$ . This pool contained  $10^7$  infectious units/ml measured as focus-forming units (FFU/ml).

#### *Virus Assay*

Infectivity was measured in eight-chambered tissue-culture slides ('Lab-Tek', Miles Laboratories). Each chamber contained  $0.1 \text{ ml}$  of diluted infectious material and  $4 \times 10^4$  A-549 cells in  $0.2 \text{ ml}$  CM, which was incubated at  $36^\circ\text{C}$  for 78 h in a moist atmosphere containing 5% carbon dioxide. After IF staining,<sup>1,2</sup> foci with 3 or more infected cells were counted and converted to FFU/ml by means of the formula: FFU/ml equals the area of the chamber ( $81 \text{ mm}^2$ ) divided by the area counted ( $0.8 \text{ mm}^2/\text{microscope field}$ ), multiplied by the mean count/field ( $n=10/\text{chamber}$ ), and multiplied by the dilution of assayed virus suspension, corrected for volume.

#### *Sucrose Gradient*

The pooled harvest from infected A-549 cells was used undiluted or concentrated 8-fold by centrifugation for 30 min at  $30\,000 \text{ g}$  at  $4^\circ\text{C}$ . Virus suspensions ( $5 \text{ ml}$ ) were layered onto a preformed linear gradient of 30% to 50% ribonuclease-free sucrose (w/v) containing  $10 \text{ mmol/l}$  HEPES buffer, pH 7.4. The gradient, on a cushion of 60% ribonuclease-free sucrose, was centrifuged at  $167\,000 \text{ g}$  for 90 min or 480 min at  $4^\circ\text{C}$  in a vertical rotor (Spinco VTi 50) and 2 ml fractions were collected. Part of each fraction was diluted 5 fold, centrifuged at  $100\,000 \text{ g}$  for 30 min at  $4^\circ\text{C}$  and resuspended in  $50 \text{ }\mu\text{l}$  CM for negative contrast staining. To assay infectious activity, each fraction was diluted 100 and 400 fold.

#### *Electron Microscopy*

Morphology of virus particles was determined by negative contrast stains of the pooled harvest from infected A-549 cells and gradient fractions with maximum infectious activity. Staining was done with either 2% potassium phosphotungstate, pH 7 (PTA) or 1% uranyl acetate pH 4.5 (UA). Immune electron microscopy was performed by the technique of Kapikian et al.<sup>8</sup> with human and rabbit antisera to Hantaan virus and appropriate normal serum controls. Equal volumes of virus-containing fluid and 1:10 dilution of antiserum were used. Volumes were  $0.05 \text{ ml}$  from gradient experiments and  $0.1 \text{ ml}$  from the supernatant culture fluids. Specimens were examined in either a JEOL JEM-100CX or JEOL JEM-100B electron microscope operating at  $80 \text{ kV}$ . Magnification was calibrated by use of a carbon replica of a grating with  $463 \text{ nm}$  spacings.

### **Results**

The pooled supernatant fluids contained round to oval particles which varied in diameter from 80 to  $110 \text{ nm}$

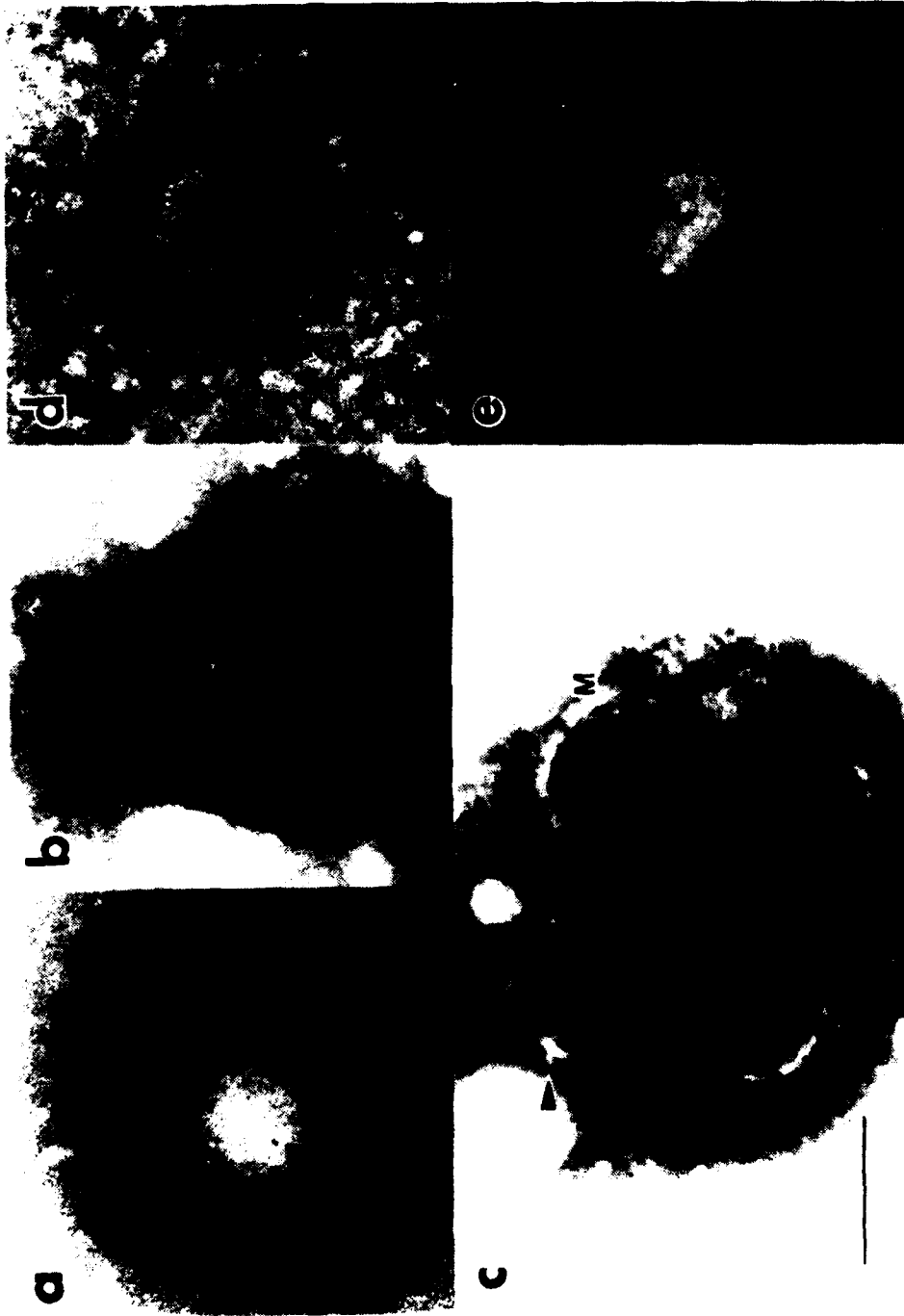
(average, 95 nm). Although ultrastructural detail was not good we could discern a peripheral structure suggestive of surface subunits in some unfixed particles (fig. 1a). The unfixed virion in fig. 1b displays putative surface projections which are hollow cylinders approximately 11 to 13 nm in diameter. Fixation with 0.5% glutaraldehyde for 72 h at 4°C resolved the virus envelope and presence of surface projections (fig. 1c). Treatment with 0.05% paraformaldehyde revealed particles unpenetrated by stain which clearly exhibited a surface substructure (fig. 1d). These subunits were regularly spaced.

To determine whether or not the observed particles were associated with virus infectivity, we attempted isolation of virus in sucrose gradients. In four separate experiments with centrifugation times of 90 min or 480 min, maximum virus infectivity was located at buoyant densities of 1.15 to 1.18 g/ml. Virions were found only in fractions at or adjacent to those with maximum infectivity (fig. 1e). The results of infectivity assays and electron microscopy carried out with coded fractions are illustrated in fig. 2. It was apparent that particles and infectivity were co-variant. No virus-like particles were seen in the dense fractions from the cushion interface or cushion, the region where reovirus or orbivirus virions should appear if present in sufficient concentration to be detected.

Aggregates of virions were observed by immune electron microscopy after reaction with immune sera but not after reaction with normal sera. One such clump produced by the anti-Lee strain rabbit serum is shown in fig. 3.

### Discussion

These data suggest that the morphology of Hantaan virus is Bunyaviridae-like rather than orbivirus-like as previously reported.<sup>6</sup> Although Hantaan virus resembles Uukuniemi virus<sup>9</sup> in having hollow surface projections (fig. 1b), there is a marked similarity to other Bunyaviridae, such as Rift Valley fever (RVF) virus. In a structural study of RVF virus, 21 equatorial subunits were observed.<sup>10</sup> None of our micrographs provided unequivocal evidence; however, from the diameter of surface projections in fig. 1b (11 to 13 nm) we calculated that there would be 23 to 27 surface subunits on the equator of Hantaan virus, and from surface projections discernible on paraformaldehyde-fixed virions (fig. 1d) we calculated that there would be 30. The most important evidence was the repeated association of Bunyaviridae-like particles with infectivity at buoyant densities of 1.15 to 1.18



**Fig. 1.—Morphology of virus particles in negative-contrast stains of pooled harvest from infected A-549 cells and gradient fractions with maximum infectious activity.**

Unfixed particles from supernatant culture fluids (a and b, PTA) are round and have substructure suggestive of surface projections (arrow). These subunits are hollow cylinders (b). Surface projections (arrow) and a membrane envelope (M) are more clearly resolved in particles that have been fixed in glutaraldehyde (c, PTA). Particles fixed in a paraformaldehyde resolve surface substructure clearly (d, U/A) because stain did not penetrate the virion. Unfixed particles from gradient fractions stain poorly but substructure can be seen (e, PTA). Scale bar, 100 nm (a-e).

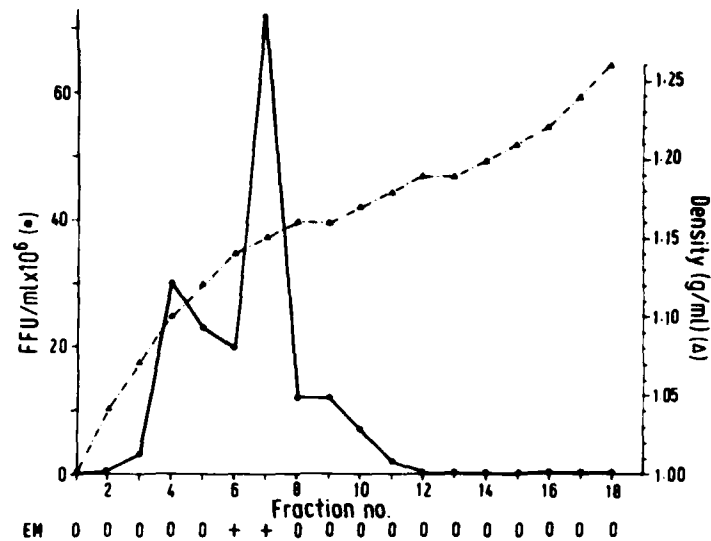


Fig. 2—Distribution of infectious virus in sucrose gradient.

Infectious activity is in FFU/ml (●—●). Zero is arbitrary because threshold for dilutions used here was  $\leq 10^5$  FFU/ml. The density of sucrose measured by refractometry is in g/ml ( $\Delta$ — $\Delta$ ). Presence (+) or absence (0) of virus particles by electron microscopy is indicated below abscissa.

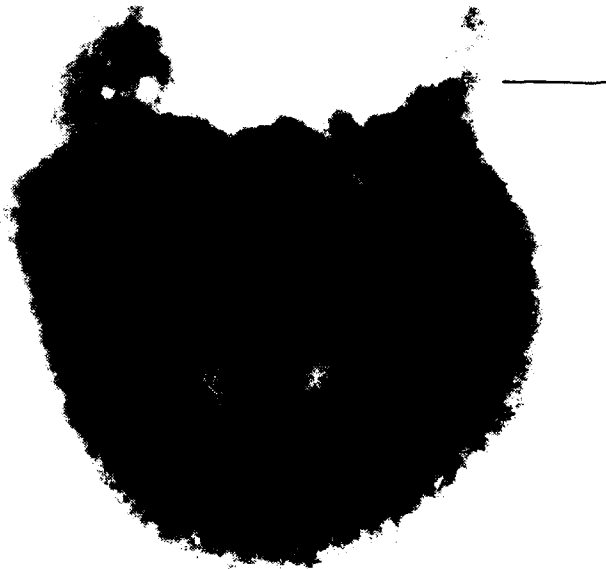


Fig. 3—Negative-contrast stain of virus particles aggregated by immune rabbit serum (PTA, scale bar, 100).

g/ml. This result overlaps with the reported range of buoyant densities for many envelope viruses, including the Bunyaviridae.<sup>11,12</sup> Further, although the techniques and reagents we used were designed to detect reovirus, no reoviruses were found. Similar results were obtained in another laboratory using plaque-purified virus derived from one of our original cell culture stocks of the 76-118 strain.<sup>13</sup> Other investigators have reported that acute and convalescent sera from patients with haemorrhagic fever with renal syndrome (HFRS) contained antibodies reactive with an antigen of similar buoyant density.<sup>14</sup> The aetiological agent of HFRS is antigenically similar, if not identical, to Hantaan virus.<sup>1</sup>

The Bunyaviridae-like morphology is consonant with our published<sup>2,3</sup> (and unpublished) data on the physicochemical properties of Hantaan virus. Final taxonomic placement of Hantaan virus depends on either elucidation of its genome and virion polypeptides or recognition of an antigenic relation with a known member of the taxon. Work in these areas continues.

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