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Electron Microscopy of Vitrified-Hydrated La Crosse Virus

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La Crosse (LAC) virions were cryopreserved by rapid freezing in a thin layer of vitreous ice. The vitrified-hydrated LAC virions were subsequently imaged at −170°C in a transmission electron microscope equipped with a low-temperature specimen holder. This cryoelectron microscopic technique eliminates the artifacts frequently associated with negative staining. Images of vitrified-hydrated LAC virions clearly revealed surface spikes as well as bilayer structure. Size measurements of the vitrified-hydrated LAC virions showed heterogeneity, with diameters ranging from 75 to 115 nm. Regardless of the particle size, the spike was about 10 nm long, and the bilayer was about 4 nm thick. The spikes are interpreted to be one or both of the glycoproteins, and the bilayer is interpreted to be the membrane envelope of the virus. In contrast to the pleomorphic appearance of the negatively stained LAC virions, the vitrified-hydrated LAC virions showed uniform spherical shapes regardless of their sizes.

La Crosse (LAC) virus is a member of the California encephalitis serogroup of the *Bunyaviridae*. This virus is a causative agent of human encephalitis. It was originally isolated from brain tissue of a patient with a fatal case of meningoencephalitis in La Crosse, Wis. (15). It has also been isolated from *Aedes* mosquito species (2). This virus has multiple copies of two glycoproteins, G1 (120 kilodaltons) and G2 (34 kilodaltons), in a 1:1 molar ratio, extending outward from the viral membrane (10). The viral membrane is acquired by budding of the nucleocapsids through membranes associated with the Golgi complex of the host cell (3). Within the membrane envelope of the LAC virion, L, M, and S segments of negative-sense RNA associate with many copies of protein N (25 kilodaltons) and a few copies of polymerase L (180 kilodaltons) to form nucleocapsids (10). Unlike some other negative-sense RNA viruses, the LAC virion lacks an internal matrix protein. This absence of protein suggests direct interaction between the nucleocapsids and the viral membrane.

Electron microscopy has been a useful tool for determination of the structure of spherical viruses (8). The most commonly used specimen preparation technique has been negative staining. Unfortunately, this procedure may cause destruction or alteration of the specimen by dehydration and chemical modification (12). A more recent procedure is ultrarapid freezing of biological macromolecules in a thin layer of vitreous ice (9). These vitrified-hydrated specimens are then examined in a transmission electron microscope equipped with a low-temperature specimen holder. This method preserves the structural detail of the virus particle in the native state in the electron microscope (1, 4, 16). This communication describes the structural features of infectious LAC virions embedded in vitreous ice as revealed by cryoelectron microscopy.

LAC virions were grown in BHK-21 cells and concentrated from the growth medium by differential centrifugation (30 min at 12,000 × *g* and 2 h at 105,000 × *g* through a 2.5-cm column of 30% glycerol in Tris-EDTA buffer [pH 7.2]). The LAC virions were purified by centrifugation in glycerol-tartarate gradients for 2 h at 210,000 × *g* (10). The purified virions were subsequently pelleted at 210,000 × *g* for 45 min and suspended in Tris-EDTA buffer containing 0.1 M NaCl. All the centrifugation steps were carried out at 4°C.

LAC virions were negatively stained by applying a drop of the viral suspension to a carbon film-coated electron microscope grid, blotting with filter paper, applying a drop of 1% aqueous uranyl acetate solution at pH 5.2, blotting again, and air drying. The following procedure was used in preparing the vitrified-hydrated specimens with no negative stain. Thin liquid layers of the viral suspension were prepared on grids covered with a holey carbon film and held by tweezers mounted in a mechanical guillotine-type plunging device (1, 9). A drop of the suspension was applied to the grid, and a piece of filter paper was pressed against it, leaving thin films of the liquid spanning the holes in the carbon film. The guillotine was then triggered, and the specimen was plunged into liquid ethane at its melting point. This entire operation was carried out in a glove box kept at a slightly negative pressure to prevent any escape of the virus into the laboratory atmosphere (T.-W. Jeng, Y. Talmon, and W. Chiu, submitted for publication). The grid was then transferred under liquid nitrogen into the work station of a Gatan 626 cryoholder and transferred in the holder into the microscope, where it was kept at about −170°C during observation. Because of the radiation sensitivity of these virions embedded in a vitreous ice matrix (5, 14), low electron doses of about 1,0000 to 1,500 electrons per nm^2 were used to record the images, usually at a magnification of ×23,000. We used Kodak SO-163 film developed for 12 min in full-strength Kodak D-19 developer at 20°C.

We measured the diameters of 350 virions from randomly chosen areas in the images of vitrified-hydrated LAC virions. Electron micrographs were digitized in a Perkin-Elmer microdensitometer with a scanning interval equivalent to 0.7 nm in the object. The digitized images were reproduced on an AED 512 graphics display terminal linked to a VAX 11/750 computer; only virions with well-defined bilayers and spikes were considered. Each virion was approximated as a circle with its circumference along the outer edge of the bilayer. The virion diameter was calculated from

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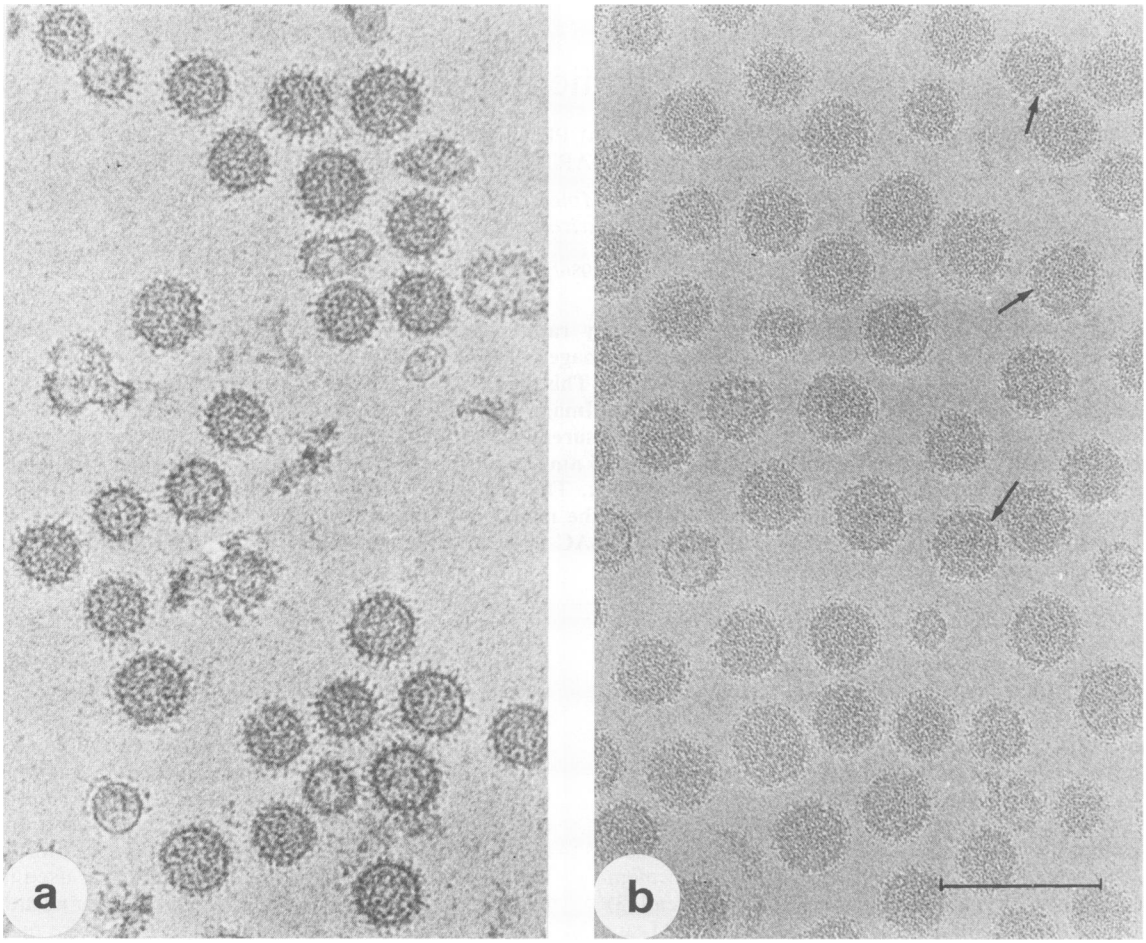

FIG. 1. Transmission electron micrographs of vitrified-hydrated LAC virions taken at (a) large defocus value at which spikes are visible, and (b) small defocus value, at which some of the membrane bilayers are visible (arrows). Bar, 200 nm.

the diameter of the circle plus the height of the surface spikes.

LAC virions were visualized first by negative staining with uranyl acetate. Negative staining and drying of LAC virus samples produced images similar to those reported previously (10). Such images suggest that the LAC virions are pleomorphic. We have found that fixation with glutaraldehyde before staining gives similar results. No periodicity was seen on the surface of the virion. This observation is in contrast to results obtained with Uukuniemi virus (17) and Punta Toro virus (11), in which glutaraldehyde fixation preserved a distinct surface lattice arrangement. This difference in results could be due to the differences in the interaction between fixation or stain and the surface proteins or to the differences in glycoprotein and peplomer composition among different genera within the *Bunyaviridae* (3).

Typical results of vitrified-hydrated LAC virions are shown in Fig. 1. Figure 1a is a highly defocused image (Δf, ~6 μm) of the virions in the vitreous ice matrix without any support film. The large amount of defocus improves the phase contrast at low resolution, enhancing details such as the surface spikes. These spikes are interpreted as one or both of the glycoproteins, which have been shown biochemically to be located at the external surface of the virions (10). Almost all of the particles in Fig. 1a have a dark line underneath the spikes. This dark line in most particles (Fig. 1b) was resolved into two lines with a 4-nm spacing when the picture was taken with a smaller defocus. This distance is consistent with that of a typical membrane bilayer (7). Therefore, we interpret this morphological feature as the viral membrane. In these micrographs, although the majority of the particles are intact, a few particles are distorted. This distortion may have been introduced during any of the preparative steps. Density differences between molecular components of the virus and the ice and proper defocus make it possible to obtain a visible level of contrast without any staining (5, 6). Images such as those of Fig. 1 give a clear picture of the glycoprotein spikes and membrane bilayers of the virions without distortions associated with staining and drying. In effect, the virions are shown in their native state without a change in the concentration, pH, or ionic strength of the solution in which they are suspended.

The uniform spherical appearance of vitrified-hydrated LAC virions refutes speculation about the pleomorphic nature of LAC virions based on the images of negatively stained virions. The negative staining may have caused distortion and collapse in the LAC virions. An unexpected finding was the distribution of sizes in the LAC virion preparation as shown in the histogram in Fig. 2. This size heterogeneity is unlikely to have been caused by flattening of the virions during the specimen preparation. When a liquid specimen is thinned down, particles larger than the thickness

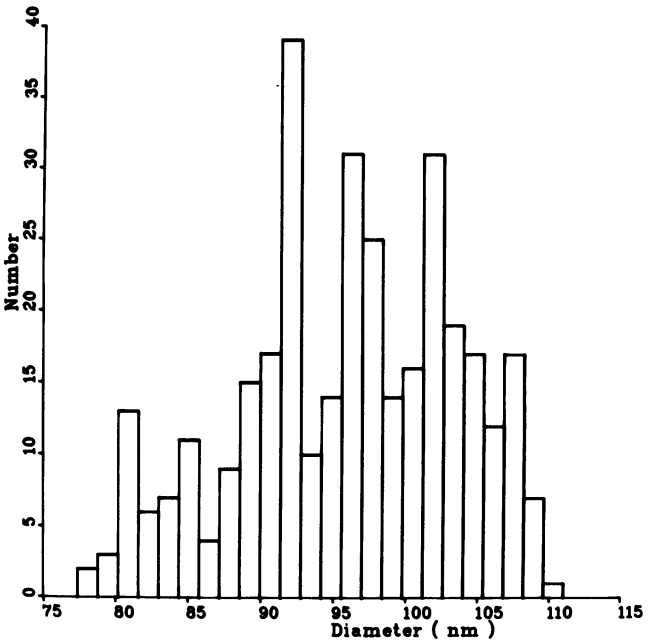

FIG. 2. Histogram of size distribution from 350 images of vitrified-hydrated LAC virions. The estimate of the virion diameter includes the glycoprotein spikes.

of liquid film are pushed to thicker areas, causing size fractionation according to film thickness (13). We have not observed such a phenomenon in this case. The biochemical basis for this heterogeneity is not yet known. Irrespective of the size of the virions, the membrane bilayer thickness was about 4 nm, and the length of the spikes was about 10 nm.

These preliminary results suggest the need for an evaluation of the biochemical activities of the LAC virions with different sizes and correlation of their biological activities with the biochemical composition. We speculate that the size difference is related to the RNA content of the virions. For example, particles containing one set of L, M, and S nucleocapsids are small, and larger-sized particles contain more than one copy of the S or M nucleocapsids or both. This difference is consistent with the observed ratios of L, M, and S RNA molecules in purified bunyavirus preparations being nonequimolar (3). To resolve the detailed arrangement of the glycoproteins on the viral surface, the three-dimensional structure of the virion must be determined. A combination of cryoelectron microscopy and image processing may lead to a quantitative description of the low-resolution molecular structure of LAC virions.

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