Rev. Biol. Trop., 42 (Supl. 2): 175-178, 1994

A rotary shadow freeze-drying replica method for the three dimensional view of virus ultrastructure

Francisco Hernández[1], Hiroshi Akahori[2], Yasuhiro Hosaka[3], Kaoru Akaike[4] and Nobuko Ikegami[4]

[1] Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica.
[2] Department of Microbiology, Osaka University of Pharmaceutical Sciences, Matsubara, Osaka 580, Japan.
[3] National Institute for Neuroscience, National Center of Neurology and Psychiatry. Ogawahigashi, Kodaira, Tokyo 187, Japan.
[4] Clinical Research Institute, Osaka National Hospital, Osaka 540, Japan.

Abstract: A new rotary-shadowing process to obtain freeze-drying replicas is described for the analysis of virus ultrastructure, using the inner capsid of human rotavirus as a model. The findings corroborate the icosahedral symmetry with an arrangement pattern of capsomers of T=13L.

Key words: Rotavirus, freeze-drying replica technique, ultrastructure.

The earliest electron microscope methodologies used to elucidate the three-dimensional (3D) arrangement of isomorphic viruses were shadow casting and negative staining. The former was abandoned because of the low resolution of the images obtained, and the latter is still widely used. Nevertheless, negative staining methods have inherent difficulties such as the alteration of fine ultrastructure due to electron damage, and Moiré images. The solutions to these problems were tackled, in the first case, by working under low electron doses (Unwin 1974, Kubozoe *et al.* 1979), and in the latter case with optical difraction techniques, which is successfully used only with filament particles or the elongated heads of bacteriophages (De Rosier and Klug 1972).

On the other hand, shadow-replica methods obviate the above mentioned troubles, because only one face of the virion particle is shadowed, then the virion is digested and only its mold is analyzed under an electron microscope. For these reasons, replicas have high resolution without Moiré images. One example of its applicability is the resolution of the controversies that emerged from the description of rotavirus ultrastructure. In that case, misinterpretation of negative-stained images leaded to reported triangulation patterns (T) of 3, 9, and 16 (Hernández and Akahori 1986). But Roseto *et al.* in 1979, using an unidirectional-shadow freeze-replica technique, reported a skew T 13 model for this agent. Their results were confirmed by the analysis of chemically (Lubert *et al.* 1986) or spontaneously disrupted virions (Hosaka *et al.* 1992) and by using cryoelectron microscopy (Prasad *et al.* 1988).

The aim of this report is the description of a rotary shadow replica method that can reveal a major area of the virion to show two or three neighboring pentamers.

MATERIAL AND METHODS

The procedure for the purification of the rotavirus was previously described by Hosaka *et al.* (1991). The purified virus was pelleted by ultracentrifugation and resuspended in dis-

tilled water. A drop of the virus suspension was mounted into a freeze-fracture holder of a metal device (Hitachi HF Z-2) covered by aluminum foil. The virus preparation was covered by another aluminum foil; thus the virus suspension was pressed between two aluminum-foil sheets. Then, this preparation was rapidly frozen on slush nitrogen. The metal block also was frozen in liquid nitrogen (LN_2) and the holder was adjusted in the metal block under LN_2, and immediately loaded into a vacuum evaporator (Hitachi HU S5). The upper aluminum foil was removed by hand before closing the chamber. The vacuum system was activated and, when it reached *ca* 10^{-6} torr, the temperature was raised to -60°C. Under these conditions, carbon was evaporated at 90° to produce a film over the viruses, and the temperature continued to raise to room temperature in approximately 2 hours. At this time, the sample was removed from the evaporator and the carbon film was covered with a drop of nitrocellulose glue (Cementine ^{R}) to protect its surface from accidentally cracking. When the glue was dry, forming a kind of helmet on the sample, it was placed in a dish with sodium hypochloride (domestic bleach) to remove the organic matter. In this step the specimen was only a carbon mold of the virus recovered by plastic; it was washed with water, air dried, and loaded again into the vacuum evaporator; but this time it was rotary shadowed with platinum at *ca.* 15° and backed with carbon. Afterwards the plastic was dissolved in acetone and the replica fragments were caught with uncovered grids and analyzed under a transmission electron microscope. Photographs of the same area were taken a tilting angle of 10°, for stereo pair study.

RESULTS AND DISCUSSION

The analysis of rotary-shadow replicas permit a clearer observation of a major area of the virions than that obtained with unidirectional shadow, because all of the exposed surface of the virion is shadowed. In an unidirectional shadow only part of the upper face of the particle can be analyzed, since the rest of the virion is not resolved and appears as a clear zone -or black in the inverted image- without details of its ultrastructure. In contrast, the image of rotary-shadow replica reveals the arrangement of the capsomers in all the upper face of the virion. The use of stereopairs to obtain three-dimensional images facilitates the analysis of the ultrastructural arrangements of capsomers. In this kind of image it is easy to identify two, and in some virions three, neigboring pentamers, as is indicated in Figure 1. This analysis corroborates the T=13 L model of the inner capsid of human rotavirus as was previously described.

The finest details of virus ultrastruture can be observed, preferably by negative staining, especially under low doses of electron. But this technique generates superimposed images -the Moiré phenomenon- that can be obviated using rotary-shadow replica techniques, because their images correspond only to the shadow face, producing one-sided image. Thus, the ideal way for the ultrastructural analysis of virus ultrastructure using conventional electron microscopes is the complementary study of negative-staining and replica methods.

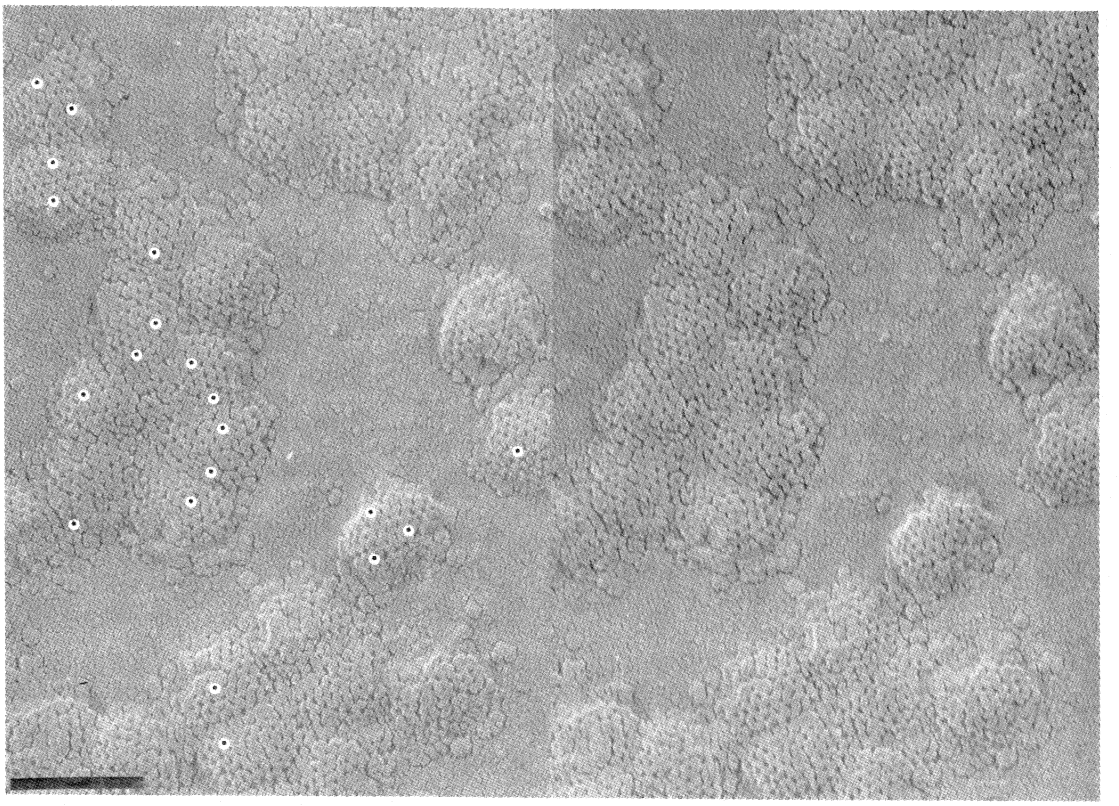

Fig. 1. Stereo pair of a rotary shadow freeze-drying replica of human rotavirus. Black and white dots indicate pentamers. Bar=100 nm.

RESUMEN

Se describe un método de sombreado rotatorio para preparar réplicas (copias) de viriones estabilizados mediante congelación y secado a baja presión. Esta réplicas permiten el análisis de la ultraestructura viral; empleándose como modelo de estudio la cápside interna de rotavirus humanos. Los hallazgos corroboran el patrón de simetría icosaédrico con un patrón de capsómeros de T=13L, previamente descrito.

REFERENCES

De Rosier, D. J. & A. Klug. 1972. Structure of the tubular variants of the head of bacteriophage T4 (Polyheads). J. Mol. Biol. 65: 469-488.

Esparza, J. & F. Gil. 1979. A study on the ultrastructure of human rotavirus. Virology 9: 141-150.

Hernández, F. & H. Akahori. 1986. Ultraestructura de rotavirus. Rev. Biol. Trop. 34:89-95.

Hernández, F., H. Akahori, Y. Hosaka, N. Ikegami, K. Akatani. 1990. The finest ultrastructural details of the inner layer of rotavirus revealed by minimal electron doses. J. Electron. Microsc. 39: 106-108.

Hosaka, Y., H. Akahori, F. Hernández, K. Akaike & N. Ikegami. 1991. Pentamer arrangements in fragments of human rotavirus inner capsids observed by low dose electron microscopy. J. Electron. Microsc. 40: 407-410.

Kubozoe, M., S. Okamura & Akahori, H. 1979. An experiment with the spot scan TEM technique. J. Electron. Microsc. 28: 263-274.

Lubert J. E. , F. Gil, F. Liparandi & J. Esparza. 1986. The structure of the rotavirus inner capsid studied by electron microscopy of chemically disrupted particles. J. Gen. Virol. 67: 1721-1725.

Prasad, B. V. V., G. J. Wang, J. P. M. Clerx & W. Chiu. 1988. Three-dimensional structure of rotavirus. J. Mol. Biol. 199: 269-275.

Roseto, A., J. Escaig, E. Delain, J. Cohen & R. Scherrer. 1979. Structure of rotaviruses as studied by the freeze-drying technique. Virology 98: 471-475.

Unwin, P. N. T. 1974. Electron microscopy of the stacked disk aggregate of tobacco mosaic virus protein. II: The influence of electron irradiation on the stain distribution. J. Mol. Biol. 87: 657-670.