NOTES

Intracellular Inclusions Resulting from Infection with African Horsesickness Virus

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Intranuclear and intracytoplasmic inclusions in monkey kidney cells infected with African horsesickness virus were studied with the aid of electron microscopy.

During experiments on the morphogenesis of African horsesickness virus (AHS; 2), intranuclear and intracytoplasmic inclusions were seen in monkey kidney cells, MS (4) and VERO (10), infected with several strains of virus. The inclusions were found in many thin sections of cells infected for 24 hr or longer, by which time virus particles were fully formed. Very often the inclusions were a clue to the presence of virus particles. In studies made with light microscopy on AHS virus in the same cell lines (8), intracellular inclusions were observed, but whether those were identical to those seen in electron micrographs is uncertain.

To determine the nature of the inclusions, MS cells infected with type 9, S2 (8) AHS virus were fixed 24 hr after infection in 1% glutaraldehyde in phosphate buffer for 20 min, postfixed in 2% osmium for 20 min, dehydrated in ethyl alcohols (G. Millonig, 19th Annu. Meeting of the Electron Microscope Soc. of Amer., J. Appl. Phys., p. 1637, 1961), and embedded in glycol methacrylate (GMA; 1). Thin sections were floated on the following enzyme solutions (11) for 2 to 3 hr: 0.5% (w/v) pepsin (2 × crystallized, Mann Research Laboratory) in 0.1 N HCl; 0.5% (w/v) trypsin (2 × crystalline; Worthington Biochemical Corp., Freehold, N.J.) in distilled water adjusted to pH 8.0 with 0.1 N NaOH; 0.3% (w/v) ribonuclease (3 × crystallized; salt-free, Worthington) in 0.1 M sodium acetate (pH 6.4); and 0.3% (w/v) deoxyribonuclease (1 × crystallized, salt-free; Worthington) in 0.1 M sodium acetate (pH 7.0) with 0.005 M MgSO₄. They were rinsed with water, stained with uranyl acetate,

and examined in an RCA-EMU-3G electron microscope.

Figures 1 and 2 show inclusions in infected cells embedded in Epon. Clusters of virus (arrows) are also visible in these selected sections. Similar inclusions (Fig. 3) show no digestion in glycol methacrylate sections treated with ribonuclease for 3 hr. However, it has previously been shown that ribonuclease removes the viral core in GMA sections (2).

When the treatment was deoxyribonuclease for 3 hr (Fig. 4), there was also no change in the lamellar structure. An experiment with sections floating on distilled water for 3 hr (Fig. 5) showed no change in the inclusions and served as a control. Digestion with trypsin (Fig. 6 and 7) and pepsin (Fig. 8 and 9) did cause a change in the inclusions. With both there was a definite decrease in the density of the lamellar structures after 2.5 hr.

Table 1 shows a correlation between the appearance of the inclusions and the infectivity of the extracellular virus in tissue culture cells. These data would indicate that the lamellae were formed at a time when the production of virus was most active and that they are a consequence of the virus infection.

These experiments reveal that the characteristic inclusions accompanying the development of AHS virus in monkey kidney tissue culture cells are proteinaceous. Both trypsin and pepsin removed them from the GMA sections, whereas the two nucleases (ribonuclease and deoxyribonuclease) had no effect. Attempts at identifying the same structures with fluorescent antibody in ordinary histological sections have been unsuccessful, since, in most cases, the inclusions are very close to the limit of resolution of the

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FIG. 1. Epon-embedded thin section of AHS virus cluster (arrows) and intracytoplasmic inclusion. Bars in this and all other figures are equal to $1\ \mu\text{m}$.

FIG. 2. Thin section, Epon-embedded, of AHS virus (arrows) and inclusion in the cell nucleus (N).

FIG. 3. Inclusions intact in both nucleus (N) and cytoplasm after 3 hr of ribonuclease treatment of GMA section.

FIG. 4. Intact inclusion near possible virus accumulation (arrow) after 3 hr of deoxyribonuclease digestion of GMA section.

FIG. 5. Control GMA section floated on water for 3 hr with no effect on the inclusion structure.

light microscope. As pointed out by Hayashi and Matsui (3), autoradiography does not necessarily confirm similar inclusions as viral antigen. Crystalline inclusions accompanying the formation of adenovirus 5 in tissue culture cells were reported by Morgan et al. (7). In a study of

BHK-21 cells infected with AHS virus (5), the presence of similar inclusions was not reported.

Similar structures have been examined in thin sections of *Datura stramonium* leaf cells infected with tobacco etch virus (3, 6). There was no evidence that the inclusions definitely contained

FIG. 6. Intranuclear inclusion in a GMA section treated with trypsin for 2.5 hr.

FIG. 7. Cytoplasmic inclusion removed by treatment with trypsin for 2.5 hr in GMA section.

FIG. 8. Intranuclear inclusion removed by pepsin treatment for 2.5 hr, whereas virus cluster retains cores (arrows); GMA section.

FIG. 9. Removal of inclusion by pepsin after 2.5 hr from cytoplasm in GMA section.

TABLE 1. *Development of extracellular AHS virus (S2) in MS cells and appearance of inclusions in thin sections*

Hr postinfection	Virus titer ^a	Lamellar structures in thin sections ^b
4	2.3	—
8	4.8	—
12	6.2	—
18	6.1	+
24	6.2	++

^a Initial multiplicity 20 TCID₅₀/cell. Virus titer is expressed as log TCID₅₀ per milliliter.

^b Lamellar structures were seen in some sections (+) and could be found in almost every ribbon (++); no lamellar structure was visible (—).

tobacco etch virus nucleic acid or protein. Inclusions associated with bean yellow mosaic virus (9) are digested, however, in GMA sections by pepsin in the same manner as are AHS-associated inclusions. The demonstration of the AHS inclusions does show that, in animal virus infection, cells may react in a similar manner as with the plant viruses.

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