Morphological Components of Herpesvirus. III. Localization of Herpes Simplex Virus Type I Nucleocapsid Polypeptides by Immune Electron Microscopy

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SUMMARY

Herpes simplex virus type 1 (HSV-1) nucleocapsids were observed in the electron microscope after their reaction with IgG's purified from the sera of rabbits immunized with the individual nucleocapsid polypeptides. The combining sites of NC1, the major capsid protein (mol. wt. 154K), were distributed over the entire capsid surface. This result provides further evidence that NC1 represents the major hexamer constituent. NC2 (mol. wt. 50K) was less widely distributed and appeared to be located at capsid vertices; that antigen may be a constituent of the pentamers or of peripentameric hexamers. One or both of NC3 and NC4 (mol. wt. 40K and 38K) were also located all over the capsid, possibly at positions interior to those of NC1. One or both may represent the intercapsomeric fibrils, hexamer-associated protein or material associated with the pericore. The locations of the other nucleocapsid polypeptides could not be determined.

INTRODUCTION

Non-enveloped nucleocapsids of the morphologically distinct herpesviruses contain five to seven polypeptide components (Gibson & Roizman, 1972; Perdue et al., 1975; Stevely, 1975; Allen & Bryans, 1976; Cohen et al., 1980). The herpes simplex virus type 1 (HSV-1) nucleocapsid, for example, contains seven polypeptides, each of which represents a unique gene product (Cohen et al., 1980). These components have mol. wt. of 154K, 50K, 40K, 38K, 33K, 26K and 12K (labelled NC1 to NC7 respectively): the HSV-2 nucleocapsid has a similar number of polypeptides differing slightly in mol. wt. (Gibson & Roizman, 1972; Cohen et al., 1980).

The capsids comprise 162 apparently hollow, prismatic capsomers arranged to form an icosahedron with a triangulation number of 16 (Wildy et al., 1960). The 150 hexamers consist of three or six morphological subunits and are connected by a matrix of intercapsomeric fibrils (Vernon et al., 1974; Palmer et al., 1975; Furlong, 1978). The 12 vertex pentamers have not been as well described, but they appear to be bound relatively loosely at capsid vertices and to be susceptible to removal or destruction by dehydration or trypsin digestion (Palmer et al., 1975; Vernon et al., 1976. 1978).
The nucleocapsids of HSV and of equine herpesvirus types 1 and 3 can be separated into ‘light’ and ‘heavy’ populations by density-gradient centrifugation; intermediate populations also occur. Light particles, which lack DNA and visible internal components, consist of only four polypeptides. Heavy particles contain the remaining polypeptides, as well, and the viral DNA within a structurally complex core (Gibson & Roizman, 1972; Perdue et al., 1975; Allen & Bryans, 1976), which is separated from the inner capsid surface by a space termed the ‘pericore’ (Roizman & Furlong, 1974).

Little information is available about the structural relationships of the individual polypeptides within the nucleocapsid. However, in spite of the fact that no morphologically recognizable capsid component has yet been isolated, circumstantial evidence would tend to implicate NC1, the major capsid protein, as the major constituent of the hexamer.

This study was undertaken in an attempt to locate the sites of the HSV-1 nucleocapsid polypeptides. Purified nucleocapsids were examined in the electron microscope after their reaction with immunoglobulins directed against each of the polypeptide components. The topographical distributions of several nucleocapsid antigens could be determined in this manner.

**METHODS**

Procedures. The purification of HSV-1 nucleocapsids, fractionation of viral polypeptides by polyacrylamide gel electrophoresis, preparation of rabbit antiserum to each polypeptide and extraction of the IgG fraction of each antiserum have been described (Cohen et al., 1980). For this study, immunoglobulins were purified from antisera to all polypeptides except NC6 and to light nucleocapsids, as well as from normal rabbit serum (NRS). NC3 and NC4 were not separable when gels were sliced, and the two were combined for injection; the antiserum is referred to as anti-NC3,4. Each antibody preparation except NRS IgG reacted positively with HSV-1-infected KB cells in immunofluorescence tests (Cohen et al., 1980), and the purified preparations contained 22 to 34 mg IgG/ml.

Electron microscopy. Three basic approaches to immune electron microscopy, each employing modifications to reported methods, were used. Nucleocapsids were diluted with sufficient phosphate-buffered saline (PBS) pH 7.2, 0.1 M-phosphate buffer pH 7.2 or water, to provide suitable dispersion when adsorbed to specimen grids. Grids with adsorbed nucleocapsids were rinsed with PBS and decorated *in situ* with antibody by (i) adding drops of diluted IgG and incubating the grids for up to 2 h at 37 °C (Milne & Luisoni, 1975) or (ii) floating grids on diluted antibody suspensions for up to 3 h at 37 °C (Yanagida & Ahmad-Zadeh, 1970). (iii) Suspended nucleocapsids were incubated with various concentrations of antibody for 1 to 3 h at 37 °C and then overnight at 4 °C (Almeida & Waterson, 1969). One-half ml suspensions were layered on to gradients consisting of 1 ml 5 % (w/v) sucrose in phosphate buffer, 1.5 ml 27 % sucrose and 2 ml 60 % sucrose in 5 ml tubes, which were centrifuged at 20000 rev/min for 30 min in a Beckman SW50.1 rotor. Visible bands were harvested by collecting drop-wise from tube bottoms or by side puncture. Nucleocapsids precipitated by anti-capsid IgG formed a flocculent white band on the top of the 60 % sucrose layer; unreacted nucleocapsids formed a blue-white opalescent band in the middle of the 27 % sucrose layer.

Samples were adsorbed to carbon-coated specimen grids, which were rinsed with 0.1 % ammonium acetate or water before being negatively stained with 1 % ammonium molybdate (AM) pH 7, or 1 % sodium phosphotungstate (PTA) pH 7. Other stains were found to be less satisfactory. Grids were examined in an RCA EMU-3H electron microscope at an accelerating potential of 50 kV.
RESULTS

Nucleocapsids treated with NRS IgG at concentrations of 100 μg/ml to 10 mg/ml were indistinguishable from unreacted nucleocapsids. In both cases, centrifugation through sucrose gradients resulted in the production of opalescent blue-white bands about half way through the 27% sucrose layer. Particles in these bands were well dispersed, although clumps containing two to about 20 nucleocapsids occurred occasionally.
When anti-light nucleocapsid IgG was mixed at a final concentration of 1·6 mg/ml with nucleocapsids, the opalescent suspension became cloudy almost immediately. After incubation, centrifugation produced a white flocculent band on the top of the 60% sucrose layer which contained massive aggregates of nucleocapsids. Dense stain accumulation prevented observation of attached IgG except at the edges of aggregates, where it appeared to coat the particles.

Virtually identical results were obtained when nucleocapsids were mixed with anti-NC1 IgG at the same concentration. In addition, when nucleocapsids adsorbed to grids were floated on anti-NC1 IgG at a concentration of 0·24 mg/ml, the particles became coated with
Immuno-E.M. of HSV-1 nucleocapsids

Fig. 3. Nucleocapsids treated with anti-NC3,4 IgG. These particles were recovered on a 60% sucrose cushion after centrifugation of capsids mixed with 2.4 mg/ml antibody. The particles are surrounded with IgG. Stained with PTA.

IgG, which usually did not obscure the spaces between capsomers (Fig. 1). It was concluded that NC1 antigenic sites were located all over the capsid surface.

Anti-NC2 IgG did not appear to precipitate suspended nucleocapsids when mixed at concentrations of 1.6 mg/ml or less; suspensions remained opalescent, and only blue-white bands were formed in 27% sucrose layers during centrifugation. When the concentration was raised to 12 mg/ml, however, the suspension became cloudy, and a flocculent band was formed on the 60% sucrose layer. Dense stain deposits between heavily aggregated particles in the flocculent band hindered the observation of antibodies; antibodies could be seen connecting capsids which occasionally were slightly separated from aggregates. When grids containing adsorbed nucleocapsids were floated on anti-NC2 IgG (2.4 mg/ml) for 2 h, antibody attached to single capsids almost always appeared to extend from vertices of hexagonal capsid images, and no IgG aggregates were seen covering the edges of individual particles (Fig. 2). Occasionally, antibody-like connections could be observed between widely separated capsids and, in most cases, at least one end of the connection appeared to be associated with a capsid image vertex. When attached antibody was aggregated between closely apposed capsids, the actual points of attachment could not be determined.

Aggregation of nucleocapsids was apparent almost immediately after mixing with 2.4 mg/ml anti-NC3,4 IgG, and a flocculent band was produced on the 60% sucrose cushion by centrifugation. Both centrifuged particles and nucleocapsids decorated in situ were coated with antibody (Fig. 3). However, these particles were subtly different from those treated with
anti-NC1 IgG in that the definition of individual capsomers on decorated capsids almost always was obscured, regardless of the instrumental focus level. Again, sites of attachment could not be determined from micrographs of connections between closely apposed particles.

Results obtained with both anti-NC5 and anti-NC7 immunoglobulins were difficult to interpret. When the IgG's were mixed with nucleocapsids at concentrations of 17 and 11 mg/ml respectively, centrifugation produced barely visible bands on 60% sucrose cushions; opalescent bands containing the bulk of material were formed in the 27% sucrose layers as well. The attachment of IgG to virus in the bands could not be determined with certainty in the electron microscope. Mixing with lower concentrations of IgG or decoration experiments did not result in any indication of precipitation or antibody attachment.

In all cases, thick stain deposits consistently hampered visualization of attachment sites and even of the antibody particles themselves. It was necessary, therefore, to base conclusions regarding antigen locations primarily on observations of particles decorated in situ.

**DISCUSSION**

The results indicate that polypeptides NC1, NC2 and either or both NC3 and NC4 are located at the nucleocapsid periphery with their antigenic sites exposed sufficiently for attachment to IgG. The coating of capsids with relatively low concentrations of anti-NC1 is consistent with the idea that NC1 represents the major component of the hexamer. This protein and its analogues (O'Callaghan & Randall, 1976; Killington et al., 1977) constitute the major nucleocapsid proteins of several herpesviruses (Gibson & Roizman, 1972; Perdue et al., 1975; Stevely, 1975; Allen & Bryans, 1976; Dolyniuk et al., 1976).

Antibody to polypeptide NC2 occupied a more restricted area of the capsid than did anti-NC1 IgG; anti-NC2 appeared to be associated with capsid vertices, possibly combined with a constituent of the pentamer or with a structural component associated with peripentameric hexamers. The peripentonal hexons of adenovirus type 2 are associated with unique, though minor, antigens of the capsid (Prage et al., 1970; Everitt et al., 1973). However, we cannot rule out the possibility that NC2 is distributed more widely on the capsid but is more accessible to antibody, for topographical reasons, near the vertices. Because disulphide bonds, which appear to be necessary for the maintenance of capsid morphology (McCombs & Williams, 1973), are responsible for a structural affiliation between NC1 and NC2 of HSV-2 capsids (Zweig et al., 1979), it is probable that NC2 is bound to NC1 within the capsid structure.

The sites of attachment of anti-NC3,4 IgG are also distributed all over the capsid. The observation that intercapsomeric spaces were obscured consistently by anti-NC3,4 suggests that the antibodies may have attached to sites slightly interior to those of NC1. Precise measurements of antibody extension length from capsid surfaces could not be made because of an inability to determine the exact attachment sites on two-dimensional projections of the capsids and folding of IgG molecules. Nevertheless, we may propose three possible locations for NC3 and/or NC4: (i) one or both of the polypeptides represent the intercapsomeric fibrils (Vernon et al., 1974; Palmer et al., 1975); (ii) one or both represent antigen closely associated with, or integral to, the hexamer; or (iii) one or both antigens reside on the exterior surface of the virus internal component. The last possibility arises because there exists stain-permeable structural material in the pericore, between the capsid and the virus core (Roizman et al., 1969). Stain-permeable particles, sometimes containing core-like structures, have been observed after denaturation of equine herpesvirus type 1 nucleocapsids with formamide (Vernon et al., 1978). If such material lines the inner surface of the capsid, it is conceivable that it can be reached by the 4 nm diam. Fab segments (Valentine & Green, 1967; Poljak et al., 1972) by penetration of intercapsomeric spaces, perhaps by a mechanism similar to those
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postulated for the reaction of Fab with internal proteins of intact polyoma virus and adenovirus (Kjellén & Pereira, 1968; Frost & Bourgaux, 1978).

The results indicate that at least one of polypeptides NC3 and NC4 is not restricted to heavy capsids, which represented only about 10% of the populations employed in this study (Cohen et al., 1980). That these polypeptides represent internal components might be inferred by their under-representation in light nucleocapsids and presence in heavy capsids, which include virus cores (Gibson & Roizman, 1972). One of them may be equivalent to protein 22a, a constituent of heavy nucleocapsids of both HSV-1 and HSV-2 (Gibson & Roizman, 1972, 1974; Heilman et al., 1979). However, this correlation has not been established experimentally.

Although anti-NC5 and anti-NC7 IgG reacted positively, albeit weakly, in immunofluorescent tests against homologously infected cells (Cohen et al., 1980), their reaction with nucleocapsids could not be established with certainty in these experiments. The simplest explanation is that the two antigens are internal components of nucleocapsids. Recent evidence suggests that a 12K phosphoprotein, which is present in HSV-1 virions and is associated with chromatin from HSV-1-infected cells, is equivalent to NC7 (Knopf & Kaerner, 1980).

The interpretation of the results was affected by difficulty in determining definitively negative results. The incubation of ferritin-conjugated goat anti-rabbit IgG serum with nucleocapsids suspected of having attached IgG did not clarify results obtained with anti-NC5 or anti-NC7 IgG’s, and the ferritin-tagged antibody frequently obscured the attachment sites of the other IgG’s (S. K. Vernon et al., unpublished observations).

Although the observation of the interaction of antibody with disproportionately large viruses is accompanied by various technical difficulties (Almeida & Waterson, 1969), immune electron microscopy has been used successfully to identify antibody-combining sites on large DNA-containing animal viruses (e.g. Norrby et al., 1969). Herpesvirus clumped by antibody was also observed by Watson & Wildy (1963) and by Rigby & Johnson (1972), but no attempt was made to localize antigenic sites.

A more precise determination of the structural positions of the HSV nucleocapsid polypeptides may depend on the development of methods permitting the sequential degradation of nucleocapsids, preferably into morphologically recognizable components whose biochemical and antigenic characteristics may be analysed individually.

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REFERENCES


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