Sequential Immunofluorescence and Infectivity Studies on the Replication of Herpesvirus Saimiri in Owl Monkey Kidney Cells

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SUMMARY

Methods are described for the preparation and authentication of a highly specific antiserum against herpesvirus saimiri (HVS) capsid antigens. The antiserum was used in immunofluorescence tests to follow the development of capsid antigens in HVS-infected owl monkey kidney cells throughout the virus replication cycle in parallel with sequential titrations of virus infectivity in both cells and medium. Fluorescence was detected as a round or oval, bright green area of staining at the centre of the nucleus which was similar in outline to the Cowdry type A inclusion seen in HVS-infected cells stained by haematoxylin and eosin. The first detection of fluorescence towards the end of the eclipse phase of the virus growth cycle, and its abolition by the treatment of infected cultures with cytosine arabinoside confirmed the identity of HVS capsid antigens as late antigens. The failure to detect fluorescence in the cytoplasm of HVS-infected cells has brought to light a conflict between the site of accumulation of virus capsid antigens as determined by immunofluorescence and the finding, by electron microscopy, of cytoplasmic immature particles in intact cells during the early stages of the virus replication cycle. The significance of this discrepancy is discussed in relation to its possible existence for other members of the herpesvirus group.

INTRODUCTION

The use of monospecific antisera for the detection of herpesvirus antigens has several clear-cut advantages; besides differentiating between structural and non-structural virus antigens, such antisera would allow a more precise antigenic comparison of individual herpesviruses and would provide a useful tool with which to investigate cell-virus interactions in both productive and non-productive infections. The preparation of monospecific antisera against structural proteins of both the envelope and capsid of mature particles has recently been described for herpes simplex virus (Powell et al. 1974; Powell & Watson, 1975) and highly specific reagents of this kind are clearly desirable for the study of other herpesviruses. Of these, herpesvirus saimiri (HVS) and the monkey lymphoma it causes (Meléndez et al. 1969a, b; Morgan et al. 1970) is important as a model for the investigation of herpesvirus carcinogenesis in general and may be useful in elucidating the potential aetiologic role of Epstein-Barr (EB) virus (Epstein, Achong & Barr, 1964) in human neoplasia.

But in contrast to the ease with which herpes simplex virus can be purified (Robinson & Watson, 1971; Powell et al. 1974; Powell & Watson, 1975), HVS was found in extensive preliminary experiments to suffer two important disadvantages in the production of purified...
virus for the fractionation of structural proteins and their subsequent use as antigens to induce specific antisera. These disadvantages were, first, that even maximally infected cultures gave only relatively low yields of virus, and second, the virus showed a peculiar affinity for cellular membrane debris which made purification unusually difficult. Alternative methods have therefore been used to produce a highly specific antiserum against HVS capsid antigens. The present paper reports the results obtained with this serum in immunofluorescence tests designed to determine the intracellular location of virus capsid antigens throughout the virus replicative cycle.

METHODS

Cells and technique of culture. Owl monkey kidney (OMK) cells of the 210 line were kindly provided by Dr L. V. Meléndez and were grown in glass roller bottles and maintained when infected in roller bottles or Petri dishes by the methods already described (Morgan, Achong & Epstein, 1973). For immunofluorescence, monolayer cultures of OMK cells were grown on 11 mm glass coverslips placed in 60 mm plastic Petri dishes in an atmosphere of 5% CO₂ in air.

Virus. Stock virus pools were prepared exactly as in earlier work (Morgan et al. 1973). High titre virus was prepared in the same way as the stock virus except that the virus was concentrated 40 times and debris was removed by centrifuging at 5000 g instead of low-speed centrifugation and millipore filtration. The high titre virus preparations were assayed in OMK cells by the plaque titration method of Daniel et al. (1971). Plaques were counted after 6 days of incubation and titres of about 2 × 10⁸ p.f.u./ml were usually obtained.

Purification of virus. Twenty roller bottle cultures of OMK cells with advanced c.p.e. 5 days after infection with 0.1 p.f.u./cell of stock virus were used to prepare each batch of purified virus. The culture medium was discarded, the monolayers were washed twice with phosphate buffered saline (PBS), pH 7.4, the cells were removed with glass beads into suspension in 60 ml of fresh PBS and were then sonicated in the same way as for the preparation of stock virus (Morgan et al. 1973); after this, coarse cellular debris was removed by centrifuging at 700 g for 15 min. The resulting supernatant fluid was centrifuged at 10000 g for 30 min, the pellet of small membrane debris was discarded and the new supernatant fluid centrifuged again at 50000 g for 1.5 h. The supernatant fluid from this fast run was kept for the preparation of virus-free soluble extract while the pellet was resuspended in 60 ml of PBS and centrifuged again at 10000 g; the resulting deposit was discarded and the supernatant fluid centrifuged further at 50000 g for 1.5 h. The final supernatant fluid from this second high-speed centrifugation was also kept for the preparation of soluble extract whilst the pellet was resuspended in 10 ml of PBS and used as purified virus.

Virus-free soluble extract. The supernatant fluids from each of the two high-speed centrifugations were pooled, concentrated at 4 °C from 120 to 40 ml through a dialysis membrane in Aquacide 1-A (Calbiochem Ltd, London) and then dialysed overnight against PBS. The resulting isotonic, soluble extract was divided into 2 ml lots in glass ampoules and freeze-dried; the ampoules were sealed and stored at −20 °C.

Production of virus capsid antiserum. Young adult, 2 to 3 kg, Californian rabbits were injected subcutaneously and intramuscularly with a total of 1 ml of purified virus emulsified in 1 ml of Freund’s complete adjuvant. The injections were given 8 times at weekly intervals and the animals were bled 1 week after the last injection; the resulting virus capsid antiserum was stored in screw cap bottles at −70 °C.

Absorption of virus capsid antiserum. One ml of antiserum was shaken with 2 × 10⁸ freshly harvested, washed, OMK cells for 1 h at 37 °C followed by 12 h at 4 °C. After this, the serum
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was clarified by centrifugation at 10,000 g for 30 min and shaken again with $2 \times 10^8$ freeze-dried OMK cells, 0.2 ml foetal calf serum (FCS), and an ampoule of freeze-dried virus-free soluble extract; these absorbing reagents were added sequentially with clarifying centrifugations at 10,000 g for 30 min in between.

**Gel diffusion test.** Agar (0.6%; Oxoid Ltd, London) in tris-HCl buffer, pH 8, was used in microimmunodiffusion tests according to the method of Crowle (1958). Freeze-dried preparations of virus-free soluble extract and OMK cells were each reconstituted in 1 ml of PBS before use in the tests. After the reagents had been allowed to react at 4 °C for 3 days in the gels, the presence of precipitation lines was sought under a dissecting microscope at 11 × magnification with dark ground illumination. Precipitation lines were carefully cut out, emulsified in 10 μl of PBS and negatively stained for electron microscopy.

**Virus particle agglutination test.** Purified virus (0.2 ml) containing $2 \times 10^9$ particles/ml was mixed either with 0.2 ml of absorbed virus capsid antiserum or with 0.2 ml of normal rabbit serum. The mixtures were shaken at 37 °C for 2 h and samples were negatively stained for electron microscopy.

**Serum neutralization test.** Absorbed virus capsid antiserum was tested for the presence of neutralizing antibodies to HVS in a standard plaque-reduction assay (Habel, 1969).

**Negative staining for electron microscopy.** A 5 μl drop of material to be examined for the presence of virus was placed on a carbon-coated copper grid and allowed to dry almost completely. A drop of 1.5% phosphotungstic acid (PTA), pH 6.5, was then added to the grid and the excess drained away on filter paper. All grids were examined in a Philips EM 300 electron microscope at an accelerating voltage of 60 kV.

**Virus particle counting.** Equal volumes of purified virus and a standard suspension of latex particles were mixed and samples of the mixture were examined in the electron microscope after negative staining. The concentration of virus was calculated as the mean ratio of virus to latex particles from counts made on several grids.

**Light microscopy.** Stock and infected cultures were examined in a Reichert inverted microscope to assess the general progress of cytopathic changes. Coverslip cultures were fixed in methanol at 6 hourly intervals after infection until complete cytolysis and stained by haematoxylin and eosin.

**Preparation of conjugated antibody.** Specific sheep anti-rabbit IgG (S-RIgG) antibody-FITC conjugate was prepared essentially by the methods of Elson, Singh & Taylor (1973). The purity of the RIgG used for sheep inoculation and the specificity of the resulting S-RIgG antibody were checked by immunoelectrophoresis (Williams & Chase, 1971). Small samples of the S-RIgG antibody-FITC conjugate were stored at −70 °C until used in the immunofluorescence tests.

**Immunofluorescence tests.** Coverslip cultures were washed in PBS followed by distilled water and fixed for 10 min in acetone cooled to −60 °C. The coverslips were air-dried and stored in sealed plastic bags at −70 °C. For the tests, fixed coverslip cultures were thawed in PBS at 37 °C and exposed to a 1/8 dilution of absorbed virus capsid antiserum for 1 h at room temperature, followed by 12 h at 4 °C. The coverslips were washed in PBS, reacted with S-RIgG antibody-FITC conjugate for 2 h at room temperature, washed again, mounted in 10% PBS in glycerol and examined in a Leitz Orthoplan fluorescence microscope with Ploem-type incident illumination, BG12 and BG38 exciter filters, and a K510 barrier filter. The same batch of S-RIgG antibody-FITC conjugate containing 0.2 mg/ml of protein with a fluorescein: protein ratio of 1:6:1 was used in all the tests. The following control procedures were included: (a) uninfected OMK cells tested with absorbed virus capsid antiserum followed by S-RIgG antibody-FITC conjugate; (b) infected OMK cells exposed to normal
rabbit serum followed by S-RIgG antibody-FITC conjugate; (c) infected OMK cells reacted with absorbed virus capsid antiserum followed by S-RIgG antibody for 2 h at room temperature and then exposed to S-RIgG antibody-FITC conjugate in the usual way; (d) virus capsid antiserum absorbed with purified virus and tested on HVS-infected OMK cells using S-RIgG antibody-FITC conjugate in the usual way. Before use in the test the virus was deposited from the serum by centrifuging at 15,000 g for 1 h.

*Cytosine arabinoside.* Coverslip cultures were infected with 10 p.f.u./cell and fed with maintenance medium containing 20 μg/ml of cytosine arabinoside (Upjohn Ltd, Crawley, Sussex, England) and were then fixed for immunofluorescence.

**Determination of infectivity during virus replication.** For each experiment, an infected Petri dish culture of OMK cells was harvested at the end of the absorption period and 12, 24, 36, 48, 72, 96, 120 and 144 h later. The medium was collected separately from the cells, passed through a 0.45 μm millipore filter and then stored at −70 °C in a tightly stoppered screw-cap bottle; the cell monolayer was gently washed twice with PBS, scraped into suspension in MEM with 20% FCS and likewise stored at −70 °C. When all the samples had been collected, the medium was thawed, diluted in MEM in serial tenfold steps, and each dilution was titrated in 3 OMK Petri dish cultures; the cells were likewise thawed, and were then sonicated, centrifuged, and filtered in the same way as the stock virus. The filtrates were diluted in MEM in serial tenfold steps and each dilution was titrated in 3 OMK Petri dish cultures.

**Infection of cultures.** Experimental cultures were infected with high titre virus adjusted to give 30, 10 or 1 p.f.u./cell. Coverslips and Petri dishes were inoculated with 10 μl and 0.2 ml respectively. The inoculum was left at 37 °C for 1.5 h to allow virus absorption and was then removed by gently washing the monolayers with PBS. After this the cultures were fed with maintenance medium.

**Experimental procedure.** In a first set of experiments, samples of each batch of purified virus were examined in the electron microscope after negative staining to determine the number and morphology of virus particles present, and to check for the absence of obvious cellular membrane debris.

In a second set of experiments, the specificity of absorbed virus capsid antiserum was compared to that of the unabsorbed serum. After partial absorption using only normal OMK cells and FCS, microimmunodiffusion tests were carried out against OMK cells, FCS, virus-free soluble extract and purified virus, and the precipitation lines which developed were examined by electron microscopy for the presence of virus particles. Fully absorbed virus capsid antiserum was likewise tested against the above reagents to assess the effect of the absorption procedures.

In parallel, virus particle agglutination tests with the absorbed virus capsid antiserum were used to see whether specific antibodies remained in the serum after absorption; the serum was also tested for the presence of neutralizing antibody. In further experiments using indirect immunofluorescence techniques, the intracellular location of virus capsid antigen was followed throughout the virus replication cycle in cultures infected with 30, 10, and 1 p.f.u./cell. Cultures infected with 1 p.f.u./cell were fixed for immunofluorescence testing at 6 hourly intervals until complete cytolysis whilst those infected with 30 and 10 p.f.u./cell were fixed at 6, 10, 12, 15 and 18 h after virus absorption and thereafter at 6 hourly intervals until the monolayer was completely destroyed. These latter sampling times were also used for cultures maintained after infection in medium containing cytosine arabinoside.

In a final set of experiments, the infectivity in cells and medium of cultures infected with 30, 10 and 1 p.f.u./cell was titrated throughout the virus replication cycle for a comparison with
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Fig. 1. Electron micrograph of purified virus containing well separated, empty, immature particles penetrated by PTA. The material is free of formed cellular debris and mature enveloped particles.

Fig. 2. Microimmunodiffusion test gel in which virus capsid antiserum (1), partially absorbed with OMK cells and FCS was reacted with purified virus (2), virus-free soluble extract (3), OMK cells (4), FCS (5) and (7), and PBS (6) at 4 °C for 3 days. The partially absorbed antiserum failed to develop precipitation lines with OMK cells, FCS and PBS. A strong line of precipitation (long arrow) shows the common identity of an antigen present both in virus-free soluble extract and purified virus whilst a weaker line (short arrow) was only found with virus-free soluble extract. No virus particles were detected by electron microscopy in either of the lines. Magnification × 7.5.

Fig. 3. Electron micrograph of immature particles of HVS agglutinated in closely packed aggregates after exposure of purified virus to fully absorbed virus capsid antiserum for 2 h at 37 °C. The details of capsid morphology are obscured by surface coating of the particles with the antiserum.

Fig. 4. Immunoelectrophoresis gel in which the purity of the RIgG (a and c) is shown by the single lines of precipitation (X) obtained against sheep anti-rabbit globulin antiserum (B and C) in comparison either with rabbit globulin (b) or normal rabbit serum (d) which gave multiple lines. The monospecificity of the S-RIgG antibody (A) is demonstrated by the single precipitation line (Y) given both with the pure RIgG and with rabbit globulin. The cathode was situated to the left of the gel.
the results of the experiments on the development of virus antigens using immunofluorescence and on the development of virus c.p.e. as observed in stained preparations.

RESULTS

Electron microscopy of purified virus

Each batch of purified virus was found to contain immature particles free of contaminating, formed cellular debris (Fig. 1); the particles were invariably penetrated by PTA (Fig. 1). Mature enveloped particles were never seen. The purified virus preparations contained between 2 and $3 \times 10^{10}$ particles/ml.

Authentication of virus capsid antiserum

Unabsorbed virus capsid antiserum gave lines of precipitation in the microimmunodiffusion tests when reacted with OMK cells or FCS, but these lines were completely removed by absorption of the antiserum with OMK cells and FCS (Fig. 2). The antiserum, when partially absorbed by exposure to these two reagents, was found to give lines in the tests when reacted against virus-free soluble extract and purified virus (Fig. 2). The stronger of these lines (Fig. 2, long arrow) showed the common identity of an antigen present in the virus-free soluble extract and purified virus, whilst a weaker line (Fig. 2, short arrow) was only found with virus-free soluble extract. Electron microscopy failed to show virus particles in either line, thus demonstrating that the virus had not migrated from the well into the gel (Fig. 2). The fully absorbed virus capsid antiserum did not give precipitation lines against any of the above four antigens.

Virus particle agglutination test

When purified virus was mixed with normal rabbit serum and examined in the electron microscope, 50 to 80 well-separated immature particles were seen in each grid square. In contrast, similar purified virus mixed with absorbed virus capsid antiserum was found to be agglutinated in closely packed aggregates, with the detailed morphology of the virus capsid frequently obscured by surface coating with antiserum (Fig. 3); single isolated particles were rarely seen in such preparations.

Immunoelectrophoresis

The RIgG inoculated into sheep was shown to be pure by the single line it gave against sheep anti-rabbit globulin antiserum (Fig. 4) in contrast to the multiple lines obtained where normal rabbit serum and rabbit globulin were used. The monospecific reactivity of the S-RIgG antibody was likewise shown when it gave single lines with the RIgG and normal rabbit serum (Fig. 4).

Immunofluorescence tests

When fluorescence was detected it appeared as a smooth or indented, round or oval area of bright green staining at the centre of the nucleus (Fig. 5 to 7). Fluorescing cells seemed to show this same staining pattern irrespective of whether they were found early or late in the virus replication cycle. The nucleus around the central fluorescing zone remained unstained and cytoplasmic fluorescence was never observed.

Cultures infected with 30 and 10 p.f.u./cell first became positive at 10 (Fig. 5) and 12 h respectively, whereas the earliest fluorescence in cultures receiving 1 p.f.u./cell was seen after 40 h (Fig. 6).

Irrespective of the infecting dose, when fluorescence first became detectable in a culture,
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Fig. 5. Photomicrograph of acetone-fixed OMK cells 24 h after infection with 10 p.f.u./cell of HVS and reacted with absorbed virus capsid antiserum followed by S-R1gG-FITC conjugate. Single cells (arrowed) and a group of 3 adjacent cells (bottom left corner of the field) contain a brightly staining, round to oval area of fluorescence at the centre of the nucleus. The nuclei and cytoplasm around the central fluorescent zones, as well as neighbouring cells in the monolayer, are unstained.

Fig. 6. Photomicrograph of acetone-fixed OMK cells 48 h after infection with 1 p.f.u./cell of HVS and reacted with absorbed virus capsid antiserum followed by S-R1gG-FITC conjugate. Two cells in the centre of the field contain a smooth, oval, intranuclear area of fluorescence which is similar in outline to the Cowdry type A inclusion body seen in HVS-infected cells stained by haematoxylin and eosin (cf. Fig. 8). The cells in the surrounding intact monolayer show no fluorescence.

Fig. 7. Photomicrograph of acetone-fixed OMK cells 24 h after infection with 30 p.f.u./cell of HVS and reacted with absorbed virus capsid antiserum followed by S-R1gG-FITC conjugate. A focus of degenerating cells is seen at the centre of the field. Each cell in the focus contains a characteristic inclusion-like area of fluorescence which is confined to the nucleus. A few surviving cells in the upper third of the field are unstained.

Fig. 8. Photomicrograph of owl monkey cell culture stained with haematoxylin and eosin 72 h after infection with 1 p.f.u./cell of HVS, showing a focus of virus c.p.e. The Cowdry type A inclusion body seen in the nuclei of degenerate cells at the edge of the focus (arrows) bears a close conformational resemblance to the intranuclear zone of positive staining seen in virus-infected cells by immunofluorescence (cf. Fig. 5 to 7).
about 10% of the cells were positive. At this stage, single cells or two or three adjacent cells were seen to fluoresce against a background of unstained normal cells (Fig. 5 and 6). Thereafter, intranuclear fluorescence spread to involve increasing numbers of neighbouring cells around the early centres (Fig. 7), presumably reflecting some asynchrony between cells in the replication cycle. Intranuclear fluorescence finally progressed to all cells in the monolayer, by 48 to 54 h in cultures infected with 30 or 10 p.f.u./cell and by 120 h in those infected with 1 p.f.u./cell.

**Immunofluorescence controls**

No fluorescence was observed either in normal OMK cells reacted with absorbed virus capsid antiserum or in infected cells treated with normal rabbit serum followed by S-RlG antibody-FITC conjugate. Where infected OMK cells were reacted with absorbed virus capsid antiserum followed by unconjugated S-RlG antibody for 2 h at room temperature, and then treated with S-RlG antibody-FITC conjugate in the usual way, the reaction was blocked and no fluorescence could be detected. Absorbed virus capsid antiserum treated with purified virus and then reacted with infected cells and tested with S-RlG antibody-FITC conjugate likewise failed to give fluorescence.

**Progress of c.p.e.**

In cultures infected with 30 or 10 p.f.u./cell, foci of pyknotic cells were first seen at 24 h after infection and at 72 h in those receiving 1 p.f.u./cell (Fig. 8). At these times, a homogeneous, eosinophilic, Cowdry type A inclusion body was first seen in the nucleus of cells in the foci (Fig. 8) and subsequently in nuclei elsewhere in the cell sheet as infection spread throughout the monolayer. C.p.e. was maximal at 48 to 72 h in cultures infected with 30 or 10 p.f.u./cell and at about 120 h in those receiving 1 p.f.u./cell.

**Sequential infectivity titrations**

The growth curves obtained from experiments where cultures had been infected with 1 p.f.u./cell showed a logarithmic phase of cell-associated infectivity beginning at 12 h after infection and reaching a peak at 72 h (Fig. 9a); infectivity in the medium entered a similar logarithmic phase, but at 24 h, and reached a slightly lower peak at 96 h (Fig. 9a). Growth curves from cultures infected with 30 and 10 p.f.u. were similar (Fig. 9b) except that there was a steeper rise in the logarithmic phase of infectious virus production with infectivity peaks in both cells and medium at 36 to 48 after infection (Fig. 9b).

**Effects of cytosine arabinoside**

Infected cultures treated with cytosine arabinoside failed to show c.p.e. or positive fluorescence at any stage.

**Serum neutralization tests**

Absorbed virus capsid antiserum, like normal rabbit serum, failed to show any neutralizing activity against HVS. The antiserum agglutinated the purified unenveloped particles (see above) but infectivity presumably resides in the enveloped particles.

**DISCUSSION**

The significance of the present work rests on the high specificity of the reagents which have been used. A high degree of purity of the virus preparation inoculated as antigen to raise antiserum is not of paramount importance, provided that its specificity is carefully controlled and authenticated by proper absorption. Thus, antiserum specificity has been
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Fig. 9. Growth curves obtained from infectivity titrations on cells (●—●) and medium (▲···▲) of OMK cultures harvested at regular intervals after infection with (a) 1 p.f.u./cell and (b) 30 p.f.u./cell of herpesvirus saimiri. The titrations were started after virus had been allowed to absorb at 37°C for 1.5 h. Each point represents the mean of the number of plaques formed in 3 OMK dish cultures at each time interval. The standard error about the mean ranged from 0.01 to 0.15.

established here by the absorption procedures which showed that antibodies to antigens other than those of the virus capsid were clearly removed by successive treatment of the serum with normal OMK cells, FCS and virus-free soluble extract, and by the absence of virus-neutralizing antibodies. Furthermore, such absorbed serum when exposed to purified virus before use against infected cells in immunofluorescence tests lost its reactivity, while control tests using normal rabbit serum or uninfected cells were invariably negative. In addition, the S-RiG antibody-FITC conjugate was highly specific as judged by immunoelectrophoresis (Fig. 4) and the abolition of its activity in the blocking test.

With regard to the nature of the antigens detected in the microimmunodiffusion tests by either unabsorbed or partially absorbed antiserum (Fig. 2), the fact that the precipitation lines did not contain virus particles showed that these could only have resulted from interaction between the antiserum and soluble, diffusable antigens. In this connection it is interesting to note that immature particles of herpes simplex virus likewise failed to migrate through a similar gel under comparable conditions (Robinson & Watson, 1971), and a highly purified herpes simplex virus preparation did not give lines of precipitation with a general antiserum against the virus-associated antigens (Robinson & Watson, 1971). In the present experiments, complete removal of the lines following full absorption with OMK cell,
FCS and virus-free soluble extract indicated that the activity remaining must have been directed only against antigens of the virus capsid, and in confirmation of this the fully absorbed virus capsid antiserum agglutinated purified virus (Fig. 3).

When considering the nature of the antigens detected in the immunofluorescence tests by the present highly specific, absorbed virus capsid antiserum it must be borne in mind that, in herpesvirus-infected cells, virus structural proteins may be found in three forms: (1) as soluble, newly synthesized proteins in the cytoplasm (Sydiskis & Roizman, 1966; Olshesky, Levitt & Becker, 1967), (2) as molecular aggregates in the nucleus (Spear & Roizman, 1968), and (3) as assembled capsids after further aggregation (Neurath & Rubin, 1971). Nevertheless, although these forms are different, experiments with pseudorabies virus have shown that the aggregates of structural virus protein in the nucleus include antigenic determinants which are also present on the surface of assembled virus particles, and that these determinants are immunologically distinct from those of precursor proteins in the cytoplasm (Fujiwara & Kaplan, 1967). It is reasonable to assume that with HVS there are likewise antigens shared both by molecular aggregates of virus structural proteins and assembled capsids, and that the virus capsid antiserum must thus have had activity to both.

In view of this, the absorbed virus capsid antiserum is considered to have been suitable for the detection and localization of newly assembled immature particles and the aggregates of precursor proteins from which their capsids were formed. Thus, the sequential immunofluorescence tests have clearly shown that virus capsid antigens were confined to the nucleus throughout the virus replication cycle (Fig. 5 to 7) and it is of interest that the central, intranuclear area of fluorescence in HVS-infected cells (Fig. 5 to 7) closely resembles the Cowdry type A inclusion body seen in stained cells by light microscopy (Fig. 8). The inclusion body-like nuclear fluorescence of HVS capsid antigens detected in the present work with an authenticated virus capsid antiserum (Fig. 5 to 7) is similar in distribution and outline to one of several antigens that have been observed with more general antisera against a wider range of virus-associated antigens in cells infected with herpes simplex virus (Roane & Roizman, 1966; Géder et al., 1967; Roizman, Spring & Roane, 1967; Géder & Váczi, 1968; Ross, Watson & Wildy, 1968). The failure to detect virus capsid antigens in the cytoplasm appears at first sight to conflict with the findings of sequential electron microscope studies on HVS which have clearly demonstrated the exclusive role of cytoplasmic immature particles in the production of immature enveloped virus during an appreciable early period of the replicative cycle (Morgan, Achong & Epstein, 1976). A similar discrepancy has recently been brought to light with the HFEM strain of herpes simplex virus where morphological evidence of cytoplasmic immature particles and their maturation by budding through cellular membranes on the one hand (Epstein, 1962; Epstein & Holt, 1963) conflicts with the findings of more recent immunofluorescence studies in which virus capsid antigens were confined to the nucleus (Powell & Watson, 1975). Further work is obviously required to resolve this discrepancy which is apparently common to several herpesvirus systems.

Although the immunofluorescence results with HVS have proved unhelpful in elucidating the significance of cytoplasmic particles seen by electron microscopy, they nevertheless have considerable importance in relation to the various phases of the virus replication cycle as defined by the one-step growth curve (Fig. 9). Thus, in the present work both the eclipse phase lasting 12 h and the latent period of 24 h have been definitively established for HVS in OMK cells (Fig. 9), since these times remained constant irrespective of the size of infecting dose above a basic threshold; a similar situation has been reported with herpes simplex virus (Kaplan, 1957; Albrecht et al., 1963; Roizman, 1969). The fact that fluorescence was detected at the end of the eclipse phase in cultures infected with 30 or 10 p.f.u./cell, clearly
identifies the capsid antigens of HVS as late antigens; for, as is well known for herpesviruses in general (Roizman, 1969), the eclipse phase represents a period of virus biosynthesis culminating in the formation of structural proteins for assembly into new virus particles. The common identity of such late HVS capsid antigens with structural virus components is also suggested by the finding that peaks of cell-associated infectious virus (Fig. 9) and the development of maximum c.p.e. coincided with the period when all cells in the culture showed fluorescence. Final confirmation that the highly specific antiserum used in the present work gave fluorescence which indeed represented late antigens was obtained in the experiments with cytosine arabinoside. This reagent is known to block late virus functions (Klein et al. 1973) and the cultures treated with it failed to show either fluorescence or c.p.e.

In addition to the results obtained on the detection and intracellular location of virus capsid antigens, the present work has also demonstrated that HVS with its extended replicative cycle is an ideal model in which to separate and explore the various phases of virus replication which may be involved in different cellular compartments; it is also shown that the highly specific authenticated antiserum is an important tool with which to pursue this. The HVS system and the antiserum have already permitted more precise comparisons to be made between the capsid antigens of HVS and those of EB virus, and such an approach is clearly applicable to similar studies on other herpesviruses.

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REFERENCES


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