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PREPARATION OF IMMUNE SERUM TO IMMEDIATE EARLY AND EARLY POLYPEPTIDES SPECIFIED BY HERPES SIMPLEX VIRUS TYPE 1

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Summary. — An antiserum to the immediate early (IE) and early (E) polypeptides specified in SIRC cells within 4 hr post infection (p.i.) with herpes simplex virus type 1 (HSV1) was prepared in rabbits by sequential injections of two different antigens. The first antigen, which contained the alpha polypeptides, was prepared from infected cells treated for 5 hr with cycloheximide and incubated in the presence of actinomycin D. The second antigen contained all polypeptides, synthesized for 4 hr after removal of cycloheximide. The ^{14}C amino acid-labelled antigen extracts, pulsed for different intervals p.i., were precipitated with the serum to IE and E antigens, with a serum to purified viral particles and control (preimmune) serum. The antigen extracts and their precipitates were analyzed by polyacrylamide gel electrophoresis. The serum to IE and E antigens reacted at least with 3 alpha polypeptides (ICP 4, 0, 11) and several beta polypeptides (ICP 6, 8, 18, 26, 36 and 39). It also reacted with the capsid polypeptide ICP 5 (155,000) which was synthesized from 3 hr p.i. Anticomplementary immunofluorescence revealed bright granular staining in nuclei of VERO and SIRC cells between 3-6 hr p.i.

Key words: *herpes simplex virus type 1*; *immediate early polypeptides*; *early polypeptides*; *polyacrylamide gel electrophoresis*; *immunoflorescence*

Introduction

The synthesis of polypeptides specified by herpes simplex virus (HSV) proceeds in a sequential order. The polypeptides formed in infected cells can be segregated into three groups designated α, β and γ according to the onset and cessation of their synthesis (Roizman *et al.*, 1974). The polypeptides of the α group are produced immediately after the removal of the cycloheximide block. The synthesis of β polypeptides depends on the presence of α polypeptides. The cellular, amanitin-sensitive RNA polymerase II transcrib-

es first the immediate mRNAs, which specify the α polypeptides (Constanzo *et al.*, 1977). The α polypeptides, in turn, are needed for further transcription. In the course of the cycloheximide block, the immediate transcripts accumulate in excess as compared to untreated infected cells (Ben-Porat *et al.*, 1974). On removal of cycloheximide, the immediate mRNA becomes associated with polyribosomes. If the cells are incubated in the presence of actinomycin D after the cycloheximide block, only α polypeptides can be produced because no further genetic information is transcribed.

Assuming that the immediate early (α), several early (β) and some structural (capsid) polypeptides are DNA-binding proteins, infected cells treated with cycloheximide and incubated either in the presence or absence of actinomycin D were extracted with a high salt buffer according to Powell and Purifoy (1976). The polypeptides, reacting with the serum obtained were described and the site of their accumulation in the nuclei of infected cells was identified by immunofluorescence.

Materials and Methods

Virus. The Kupka strain of HSV 1 (isolated by Dr. R. Benda, Prague) was used throughout; it caused rounding of cells but formed no polykaryocytes. It was passaged in SIRC (Serum Institutet Rabbit Cornea) cells. Stock virus of high titre was prepared by inoculation of the monolayer with 0.5-1 PFU per cell. When showing a moderate cytopathic effect, the cells were scraped off from the glass, collected in a minimal amount of Eagle's basal medium (BEM) containing 5% inactivated calf serum (ICS), sonicated (3 times for 20 seconds at 9 kc) and stored at −70 °C. The titre of the virus suspension was 1.6×10^9 PFU/ml.

Cells. Viral antigens were prepared in SIRC cells which were grown in BEM supplemented with 10% ICS, glutamine and antibiotics in 1000-ml Roux bottles. Immunofluorescence was performed in SIRC and VERO cells grown in a similar medium. After infection, cells were kept in the same medium supplemented with 5% ICS.

Preparation of the immediate-early and early antigen extracts. Immediate-early antigens (α-polypeptides) were produced in SIRC cells. Five 1000-ml flasks with SIRC cell monolayers were infected with 200 PFU per cell; the inoculum in each flask was diluted to 50 ml in BEM containing 5% ICS and 100 μg cycloheximide per ml. After 5 hr, the cycloheximide was removed and the cells were washed with phosphate buffered saline (PBS) and supplied with fresh medium containing 2 μg/ml actinomycin D for 4 hr. *Early antigens* (α and β-polypeptides) were prepared in a similar manner, but the 4 hr incubation proceeded in actinomycin D-free medium. Cells treated in both ways were frozen and thawed, scraped off from the glass, collected into a minimal volume (4 ml per flask) of 0.02 M Tris-HCl buffer, pH 7.5, containing 0.05% bovine serum albumin, and 2 mM 2-mercaptoethanol (Powell *et al.*, 1976). Cells were disrupted by sonication (9 kc, 3 × 40 seconds). An equal volume of high salt buffer was then added to obtain a final concentration of 1.5 M NaCl and 5 mM EDTA. The mixture was allowed to stand for 40 min at 4 °C and centrifuged for 20 min at 30 000 × g and at 4 °C. The supernatant was dialysed overninght at 4 °C against 0.02 M Tris-HCl buffer, pH 7.2, containing 2 mM 2-mercaptoethanol, 2 mM EDTA, 50 mM NaCl and 10% (v/v) glycerol. The supernatant was finally centrifuged at 100 000 × g for 60 min, concentrated by Sephadex G-200 to a volume of 2 ml/flask and stored at −70 °C. The protein concentration of the supernatant was 2.6 mg/ml.

Labelled antigens were prepared using ^{14}C-amino acid hydrolysate (specific activity 1000 mCi/g) as follows: *A* — cycloheximide-treated infected cells were incubated with actinomycin D and labelled for 4 hr (10 μCi/ml); *B* — infected cells were labelled for 4 hr (10 μCi/ml); *C* — cycloheximide-treated uninfected cells were labelled for 4 hr (10 μCi/ml); *D* — infected cells were labelled with 20 μCi/ml amino acids between the 2nd and the 3rd hr p.i. and chased till the 4th hr in the label-free medium; *E* — infected cells were labelled from the 4th till the 9th hr p.i. (10 μCi/ml);

and *F* — uninfected cells were labelled for 5 hr (10 μCi/ml). The labelled cells were pretreated with BEM/10 (ten times reduced amino acids, supplemented with 2% foetal calf serum). To prepare the extracts, the cells were processed in a similar manner than the unlabelled ones.

Preparation of antiserum to IE and E antigens. The IE and the E antigen extracts were administered to 3 rabbits (4 mg/protein per injection) in the following order: IE antigen with complete Freund's adjuvant intramuscularly and intraperitoneally on days 1 and 14; E antigen with complete Freund's adjuvant on days 28 and 42; and on day 49 the last injection of IE antigen was administered intravenously with no adjuvant. The animals were bled 7 days after the last booster.

Preparation of antiserum to structural (virion) antigens. The virus was purified from SIRC cells (20 flasks) infected with 5 PFU/cell as described (Matis *et al.*, 1975). Infected cells were resuspended in distilled water and sonicated. The clarified virus suspension was subjected to two cycles of high (60000 × g, 50 min) and low (2000 × g, 25 min) speed centrifugation and further purified through a discontinuous Ficoll gradient prepared by overlaying equal volumes of 40, 30, 20 and 10% (w/w) Ficoll solutions in water. After centrifugation (25000 rev/min, 45 min, 4 °C, Spinco SW 39L rotor) the nucleocapsids and virions at the interfaces between 10—20 and 20—30% of Ficoll solutions were collected, diluted with water and pelleted (60000 × g, 60 min). Two rabbits (Nos 105 and 106) were immunized by 3 repeated intramuscular injections (days, 28 and 45) and bled 7 days after the last administration of the antigen.

Immunoprecipitation. The labelled antigen extracts (A, B, C, D, E, F) were precipitated with a mixture of the three immune serum samples to IE and E antigens, with the serum to purified virions and with preimmune serum. For each test, 20 μl of appropriate antigen was mixed with 20 μl of antiserum. After adding 50 μl PBS, the mixture was allowed to stand for 2 hr at room temperature. Thereafter, 700 μl of anti-rabbit IgG was added in a concentration of 20 mg/ml. The mixture was incubated overnight at 4 °C and centrifuged at 10000 × g for 15 min. The pellet was washed in PBS and dissolved either into the scintillation fluid or solubilized in sodium dodecyl sulphate (SDS) and 2-mercaptoethanol for polyacrylamide gel electrophoresis.

Radioimmunoprecipitation and polyacrylamide gel electrophoresis (PAGE). The pellets were dissolved in toluene ethanol scintillation fluid (three parts of toluene containing 6.6 g POP and 0.5 g POPOP per liter to two parts of ethanol). The radioactivity was measured on a Packard liquid scintillation counter. Slab electrophoresis and staining was carried out according to Spear and Roizman (1972). Slab PAGE was performed in a discontinuous buffer system with 0.1% SDS. The stacker and separation gels contained 3% or 8% acrylamide, respectively. For autoradiography, the gels were dried on filter paper and placed in contact with X-ray film. The following proteins were used for molecular weight determination: β-galactosidase, lactoperoxidase, albumin and chymotrypsinogen.

Immunofluorescence. SIRC and VERO cells were grown on coverslips. Monolayers (about 1×10^6 cells per coverslip) were infected at a multiplicity of 20—40 PFU/cell in 0.5 ml volumes. The medium was changed after 60 min of adsorption. At 2, 3, 4, 6, 8 hr p.i. (30 min was calculated for adsorption), the coverslips were removed, washed in PBS and fixed in cold acetone. Infected and unifected cells were stained with preimmune serum, with a mixture of sera to IE and E antigens and with serum to purified virions, All sera had been adsorbed to suspensions of either SIRC or VERO cells. The conjugate to rabbit IgG (SwAR, SEVAC, Prague) was fractionated on DEAE-cellulose; the fraction revealing a molar fluorescein isothiocyanate (FITC): protein ratio of 1.3 was used in a dilution of 2 mg/ml. For anticomplementary fluorescence, goat IgG fraction to guinea pig C3 was labelled with FITC. This conjugate with a molar FITC: protein ratio of 2.3 was used in a concentration of 1 mg/ml.

Fluorescent antibody staining was performed by both indirect and anticomplementary methods. Cells were stained with 4 appropriate dilutions of each serum for 30 min at room temperature, washed in PBS, overlaid with the anti-rabbit conjugate (30 min, room temperature), washed and mounted into Elvanol. For the anticomplementary method, the cells were stained with 3 appropriate dilutions of each serum (45 min, 37 °C). After washing in PBS, the cells were overlaid with guinea pig complement (2 units per 0.1 ml, diluted in PBS for 45 min at room temperature and washed again in PBS. Then the cells were stained with the anti-C3 conjugate (40 min, room temperature) and with the anti-rabbit conjugate (overnight, 4 °C). After final washing for 2 hr in PBS, the coverslips were mounted into Elvanol. A set of coverslips was stained with the sera at the lowest dilution (1 : 5) and complement was allowed to react with the anti-C3 conjugate only and an another set only with the anti-rabbit conjugate, excluding the anti-C3 conjugate.

Table 1. Radioimmunoprecipitation of early HSV antigens

Antigen	Serum	Total count/min	Sediment count/min	Sediment % of total
AG 5 cy/4	Mixture of preimmune sera 154 A, 164, 165 A	43 260	9 300	21.5
	Immune sera			
	154 B	61 880	34 370	55.5
	164 B	36 020	15 160	42
	165 B	39 490	16 680	42.2
SIRC/^{14}C	Immune serum			
	154 B	61 910	12 350	19.9

AG 5 cy/4: SIRC cells infected with HSV 1 (200 PFU/cell), incubated for 5 hr in the presence of cycloheximide (100 μg/ml) and labelled for 4 hr with ^{14}C amino acids (10 μCi/ml) in the presence of actinomycin D (1 μg/ml).
SIRC/^{14}C: uninfected cells labelled for 4 hr with ^{14}C amino acids.

Results

Radioimmunoprecipitation

The sera of the three immunized rabbits (Nos 154, 164, 165) reacted with the IE antigen (prepared by 5 hr treatment with cycloheximide and 4 hr incubation in the presence of actinomycin D) in the radioimmunoprecipitation test (Table 1.) The sediment, which was precipitated with excess of antibodies (swine serum to rabbit IgG) contained 42—55.5% of the radiolabelled material as compared to 21.5% of radioactivity present in the precipitate with preimmune serum. When uninfected labelled cells were precipitated with the antiserum, about 20% of the radioactivity was present in the sedimented material, indicating the range of nonspecific precipitation.

Polyacrylamide gel electrophoresis

The PAGE profiles of the antigens prepared in the presence and absence of actinomycin D and the profile of the control (uninfected) cell extract are shown in Fig. 1. The only virus-specific polypeptide, clearly visible in the extract from cells labelled for 4 hr in the presence of actinomycin D after 5 hr cycloheximide treatment (Fig. 1-I) is the ICP 0/8 (approximate mol. wt. 136000). In the extract from infected cells labelled for 4 hr in the absence of actinomycin D several infected cell polypeptides (ICP) of the beta group (ICP 6, 8, 18, 36 and 39) were present (Fig. 1-II). In addition, the capsid protein ICP 5 (mol. wt. 155000) was also detected.

When these two antigens were precipitated with the serum to IE and E antigens, their PAGE profiles were different (Fig. 2-I, III). The cell extract

from the material A revealed 2 polypeptides, the ICP 0/8 and ICP 11 (mol. wt. 110000). The precipitate of the extract from cells untreated with actinomycin D showed nearly all polypeptides present in this particular antigen (ICP 5, 6, 8, 11, 36). We concluded, therefore, that the serum to IE and E antigens reacted also with the major capsid polypeptide (mol. wt. 155000). This was confirmed by allowing the IEE antiserum to react with the extract from cells labelled from 4 to 9 hr p. i. (Fig. 3-I, II). Comparing the precipitates Bp IEE (Fig. 2-III) and Ep IEE (Fig. 3-II), it can be seen that they reveal the same lines except the IE protein ICP 11. Thus the synthesis of beta polypeptides continued after 4 hr p. i.

In a further experiment, the extracts from infected actinomycin D-treated and untreated cells and the control cell extract were again precipitated with anti-IEE serum, serum to purified virions and control serum. The corresponding precipitated and unprecipitated extracts were compared. The actinomycin D-treated cell extract showed the same two alpha polypeptides as described above (Fig. 4-I, II). The position of the ICP 11 line helped to identify this protein in the original material (arrow). The precipitate of the untreated cell extracts labelled for 4 hr p.i. showed proteins similar to those on Fig. 2-III except the absence of ICP 5 capsid polypeptide (Fig. 5). Nevertheless, ICP 5 was present in the unprecipitated material, but probably in a small amount invariably detecable with the serum to IEE proteins (Fig. 5). This was confirmed by the precipitation of the antigens B, D and E with serum to purified virions, This serum regularly detected the ICP 5 only in the extract from cells labelled from 4 hr p.i., but not in the extract from cells labelled until the 4th hr p.i. This indicates that at least two factors may determine the outcome of immunoprecipitation: the amount of the protein present in the cell extract and the avidity of the antiserum to the particular antigen.

When the proteins formed from the 2nd to the 3rd hr p.i. were analysed before and after precipitation with the antiserum to IEE antigens (Fig. 6), only a single IE protein, ICP 4 (mol. wt. 175000) was seen in addition to some beta polypeptides (ICP 6, 8, 36). The different patterns of the PAGE profiles of the precipitates ApIEE (Fig. 4-II) and DpIEE (Fig. 6-I) may be explained, in part, by different kinetics of IE protein synthesis in untreated infected cells as compared to the drug-treated infected cells.

Immunofluorescence

VERO and SIRC cells were infected at a high multiplicity (40 PFU per cell). Samples were taken at 2, 3, 4, 5 and 6 hr p.i. together with uninfected cells and stained with preimmune serum and antisera to IEE antigen and virion antigens by both indirect and anticomplementary procedures. In VERO cells stained with the serum to IE and E antigens, fine granular florescence was seen in the nuclei since 3 hr p.i. (Fig. 7). The nucleoli of these cells remained devoid of fluorescence. The same cells stained with the serum to purified virus by the indirect technique showed bright fluorescent granules in the cytoplasm since 5 hr p.i. (Fig. 8).

In SIRC cells the antiserum to IEE antigens stained fine and coarse granules in the nuclei from 4 hr p.i. (Fig. 9). Faint positive florescence was seen in the cytoplasm of a few cells revealing strong intranuclear florescence. The cells stained by the indirect technique with the serum to structural virus antigens showed positive cytoplasmic fluorescence at 6 hr p.i.

Anticomplementary staining with the serum to IEE antigens revealed the presence of these antigens in the nucleus, while antigens reacting with the serum to purified virions were located predominantly in the cytoplasm. The addition of complement seemed to be essential for demonstration of the IEE antigens. In our hands, however, quite satisfactory fluorescence was seen in cells stained with the anti-IgG conjugate instead of the anti-C3 conjugate. Nevertheless, the best results were achieved with a combination of both conjugates.

Discussion

The first immediate polypeptide described in HSV1-infected cells was the ICP 4, a high molecular weight protein (mol. wt. 175000). It was the major component synthesized in human embryonal lung cells infected with the DNA-negative ts mutant B2 at nonpermissive (39 °C) temperature (Courtney and Melnick, 1974). In immunofluorescence assay, the anti-VP 175 serum reacted only with the nuclei of infected cells. The overproduction of this polypeptide was stimulated by defective interfering particles of HSV (Murray et al., 1975). A similar high molecular weight polypeptide was also described in cells infected with HSV 2. It was designated ICP 5-8 and its mol. wt. was found to be approximately 182000-186000 (Powell and Courtney, 1975).

It was shown later that at least three polypeptides accumulate in HEp-2 cells starting with 90 min p.i. These three earliest virus-specific polypeptides are the ICP 4, ICP 0 and ICP 27 (Pereira *et al.*, 1977). In addition, another 2 or 3 IE polypeptides were reported to be synthesised in cycloheximide and actinomycin D-treated BHK 21 cells during the first 2 hr p.i. Their physical map locations were determined by the analysis of DNA fragments of 17 intertypic recombinants obtained from 3 ts mutants and 2 wild type strains of HSV 1 and HSV 2 (Preston *et al.*, 1978). We aimed to compare our findings with those of the authors cited and with the list of DNA-binding proteins presented by Powell and Purifoy (1976). Such a comparison may be tedious because of different cells, labelling techniques and differences in the gel concentration used.

The ICP 0 described by Honess and Roizman (1974) seems to correspond to the ICP 136000 (or 132000) rather than to the ICP 11 (ICP 110000). Both these polypeptides, ICP 110000 and the ICP-136000 were present in the cycloheximide and actinomycin D-treated antigen extract indicating that they are IE DNA-binding proteins. The IE polypeptide ICP 110000 disappeared from cells by 3-4 hr p.i.; on the other hand, a protein of similar molecular weight to ICP 0, designated ICP 8, continued to be present until

9 hr p.i. It is not clear whether the two polypeptides ICP 0 and 8 are identical. The mRNA for the IE polypeptide ICP 136000 was found to map in the region Hpa I f, a fragment situated at a distance of $50-60 \times 10^6$ daltons in the L segment of the HSV DNA (Clements *et al.*, 1977). The corresponding polypeptide in HSV 2-infected cells designated VP 143000 is an early non-structural protein, which can be demonstrated by immunofluorescence in the nuclei of infected cells by 4 hr p. i. and in the cytoplasm of HSV 2-transformed cells (Flannery *et al.*, 1975).

The high molecular weight IE polypeptide ICP (ICP 175000) was not seen in the precipitate from cells incubated in the presence of actinomycin D but was found between 2-3 hr p.i. Alternatively, ICP 26, an E polypeptide coded by the same areas of the inverted S_{ac} segments of HSV genome, was identified in the immunoprecipitate of the antigen extract labelled between 4-9 hr p.i. This polypeptide should have been present in the IEE antigen used for immunization. Assuming that ICP 26 corresponds to ICP 68000, ICP 27 would be identical with ICP 63000 IE polypeptide. The last early protein, referred to as ICP 87000, could be identical with ICP 18 or 19.

The rest of the E polypeptides, which were seen to be synthesised until 4 hr p.i., are usually referred to as beta polypeptides (ICP 6, 36, 39). They all are DNA-binding proteins. Our serum to IE and E proteins reacted at least with 10 out 12 polypeptides described as DNA-binding proteins (Powell and Purifoy, 1976). ICP 6 (mol. wt. 146000) comigrates in PAGE with the purified virus-coded DNA polymerase (approximate mol. wt. 150000). It is probable that ICP 6 is a degraded inactive form of this enzyme (Powell and Purifoy, 1977). The only γ-polypeptide, found to be present in the E antigen extract, is the capsid polypeptide ICP 155000. This is in agrement with the description of Fenwick and Roizman (1977) and Pereira *et al.* (1977), who found the onset of the synthesis of this protein between 2-3 hr p.i.

Our immunofluorescence findings showed that the IE and E proteins are predominantly situated in the nucleus. According to Pereira *et al.* (1977) they are present in a relatively small amount in the cytoplasm and are quickly transported to the nucleus. The nuclear fluorescence seen at early intervals p.i. in cells stained with our serum had two components: the IE and E polypeptides and the ICP 5 capsid polypeptide, which is also a DNA-binding protein (Powell and Purifoy, 1976). Experiments are in progress to prepare sera reacting exclusively with the α polypeptides but not so with other early polypeptides, in order to distinguish between the antigenic components of the early intranuclear fluorescence in HSV-infected cells.

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Explanation of Figures (Plates VI—IX):

Fig. 1. PAGE profiles of the IE (I), E (II) and uninfected control (III) antigen extracts labelled with ^{14}C amino acids (for details see Materials and Methods).
Fig. 2. PAGE profiles of the IE and E antigens precipitated by the antiserum to IE and E antigens (I and III, respectively). II — supernatant after sedimentation with anti-rabbit serum.
Fig. 3. PAGE profiles of the extracts from cells labelled between 4-9 hr p.i. (I) and control cells labelled for 5 hr (III) and their precipitates with the serum to IE and E antigens (II and IV, respectively).
Fig.4. PAGE profiles of unprecipitated and precipitated cell extracts.
I — IE antigen containing the α polypeptides (cycloheximide 5 hr, actinomycin D 4 hr)
II — The same antigen precipitated with antiserum to IE and E antigens
III — Control antigen
IV — Control antigen precipitated with antiserum to IE and E antigens
V — Control antigen precipitated with control serum
Fig. 5. PAGE profiles of the extract from cells labelled for 4 hr p. i. (B)
I — Untreated extract
II Extract precipitated with serum to IE and E antigens
III — Extract precipitated with serum to virus particles
IV — Extract precipitated with control serum
Fig. 6. PAGE profiles of the extract from cells labelled between 2-3 hr p.i. (D, II) and precipitated with the serum to IE and E antigens (DpIEE, I)

Fig. 7. VERO cells, at 4 hr p.i. with HSV 1. Fine granular fluorescence of the nuclei in most cells, faint fluorescence in the cytoplasm of a few cells. Serum to IE and E antigens, anticomplementary method, ×400.

Fig. 8. VERO cells, 6 hr p.i. Granular cytoplasmic fluorescence, nuclei mainly devoid of antigen. Indirect method, ×500.

Fig. 9. SIRC cells at 6 hr p.i. Bright coarse granular fluorescence in the nuclei of most cells; some cells also show positive cytoplasmic fluorescence. Serum to IE and E antigens, anticomplementary method, ×600.