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Immunoprecipitation of Herpes Simplex Virus Type 1 Antigens with Different Antisera and Human Cerebrospinal Fluids

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With 6 Figures

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Summary

Rabbit convalescent and hyperimmune sera, human patient and blood donor sera, as well as cerebrospinal fluids of humans with herpes simplex virus encephalitis all recognize similar major antigenic components in herpes simplex virus infected rabbit or human cells as shown by electrophoretic analysis of immunoprecipitates. Besides the main glycoproteins with an apparent molecular weight of 100,000 (peak I) the antisera precipitate glycoproteins in a region of an apparent mol. wt. of 60,000—80,000 (peak II), which were resolved into distinct glycoprotein species only by antibody-containing cerebrospinal fluids. The peak II glycoproteins appear on the surface of the infected cell early, and absorb neutralizing antibodies, whereas the peak I glycoproteins are less accessible. Both antigens can be demonstrated in the cell as early as about 2 hours post infection. All major antigenic components studied were found to be glycosylated except one protein with an apparent mol. wt. of 110,000. The herpesvirus specificity of these antigens is demonstrated by a variety of control experiments. The antigens detected are virion components.

Introduction

The large number of herpesvirus specific proteins, several of which are glycosylated (10, 12, 34), complicate the analysis of antigenic structures in the herpesvirus system. Besides the characterization of different herpesvirus antigens by classical serological techniques (8, 21, 36) recent attempts have been made to isolate virus-specific components and to attribute functional significance to them (9, 11, 22, 28, 29, 33). Nevertheless, there is no precise information about the molecular nature of those HSV-1 specific proteins which give rise to the biologically important antibodies in natural herpesvirus infection.

In this investigation we have used a variety of antisera, as well as human cerebrospinal fluids (CSF), to characterize the antigenic components specific for

herpesvirus infection which are detectable by the corresponding antibodies. Our results indicate that only a limited number of glycosylated proteins are preferentially recognized by herpesvirus specific antibodies. Only these proteins are more or less accessible on the surface of infected cells.

Materials and Methods

Viruses

The HSV-1 prototype strains used were KOS, XIII, and H_4, passaged 3-6 times in this laboratory. A fresh isolate (NIC) from a herpes labialis, serologically typed as HSV-1 in this institute, was passaged twice in rabbit kidney (RK) cells. Another isolate, No. 1332, passage 3, was used only for antiserum production (see below). Pseudorabies (PsR) virus was strain DEK and the infectious bovine rhinotracheitis (IBR) virus was strain RF. The infectivity titers attained by these strains on rabbit cells ranged from 5×10^6 to 8×10^7 PFU/ml. In human cells the HSV-1 strains reached titers of about 5×10^7 PFU/ml. Origin and characterization of the viruses can be found elsewhere (11, 17). Growth properties and characterization of bovine herpes mammillitis (BHM) virus, strain TVA, have been reported (3).

Cells and Infectivity Assays

Preparation and maintenance of primary RK cells, propagation of virus and infectivity tests by plaque assays have been detailed elsewhere (18, 20). RK 13 cells and a line of human embryonic oligodendroglia cells (kindly supplied by Y. Iwasaki and H. Koprowski, Philadelphia) were cultured in plastic petri dishes or roller bottles ($50 \times \varnothing 10$ cm) in Eagle's medium containing 10 per cent fetal calf serum.

Antisera and Cerebrospinal Fluids

The animal sera are listed in Table 1. Rabbit hyperimmune sera were prepared by the inoculation of virus in Freund's complete or incomplete adjuvant into the axillary or inguinal lymphnode regions (see Table 1), whereas the convalescent sera were produced by either corneal scarification or injection of infectious virus. Three weeks after booster injections (see Table 1) the rabbits were bled.

Human patients sera were used as pools (disease history in brackets): a) GP pool, consisting of 5 sera (stomatitis, "exanthema", meningitis), b) ROS pool, 5 sera (herpes labialis, herpes genitalis, meningitis, "exanthema"), c) URS pool, 7 sera (meningoencephalitis, "exanthema", herpes labialis, herpes genitalis and "infection of the inner ear") and as individual sera: 1. ELL (herpes labialis), 2. UHL ("infection of the inner ear"), 3. JAK (recurrent herpes facialis). These sera showing 80 per cent plaque reduction titers $1: \geqslant 200$ against HSV-1 (strain KOS), were kindly supplied by H. Bauer from this institute. In addition, blood donor sera (neutralization titer $1: \geqslant 100$) were investigated. The negative serum used (RR-1) had a neutralization- and CF-titer $1: \leqslant 5$.

Cerebrospinal fluid MÖB (kindly supplied by B. Fleckenstein and H. Zur Hausen, Erlangen) was obtained from a patient with encephalitis 6 weeks after isolation of HSV-1 from the CSF. Four further CSFs from patients with herpes infections of the central nervous system were kindly furnished by H. Becker, Giessen, and G. Enders-Ruckle, Stuttgart. Two CSFs from patients with non-herpetic encephalitides, with 700/3 and 70/3 lymphocytic cells, respectively, and no titers in neutralization- and CF-tests against HSV-1, were kindly supplied by V. Koester, Giessen.

Antiserum against purified human IgG and bovine IgG was made by boosting rabbits 3 times with 10 mg of IgG in incomplete Freund's adjuvant after an initial subcutaneous injection of IgG in Freund's complete adjuvant. Antiserum to purified rabbit IgG was raised in a sheep following essentially the same procedure. The IgG had been purified by a Sephadex G-200 column after ammonium sulfate precipitation using standard procedures. Because the antibodies were made against heavy and light chains of IgG we cannot exclude that they also recognize the light chains of other immunoglobulins.

All antisera were stored at $-30°$ C in active form and inactivated (30 minutes, 56° C) only for CF-assays.

Absorption of Sera

Infected or mock-infected cells were washed five times with prewarmed PBS, gently scraped off the glass, pelleted (5 minutes, $800 \times g$), resuspended in antiserum in a proportion of 1×10^7 cells per 0.1 ml and kept on ice 1 hour under gentle agitation. After a second such absorption with fresh cells the serum was centrifuged (5 minutes, $800 \times g$), followed by centrifugation of the supernatant for 10 minutes at $3000 \times g$, and finally by ultracentrifugation (Ti40 rotor, 2 hours, 30,000 rpm) to eliminate all debris and any released virus.

Complement Fixation (CF)-Test

The HSV-1 antigen used was prepared as previously described and the assay performed following a standard microprocedure (20).

Virus Purification

The virus was labelled with ^{3}H-glucosamine (40 μCi/ml) or a ^{3}H-amino acid mixture (45 μCi/ml) or ^{3}H-thymidine (10 μCi/ml). Optimal results concerning purity were obtained by the following procedure: the supernatant medium of an infected oliogodendroglia cell roller culture (approximately 5×10^8 cells) was centrifuged for 10 minutes at $800 \times g$ and the supernatant subjected to a further centrifugation for 10 minutes at $10{,}000 \times g$. The virus was sedimented through a sucrose solution of 30 per cent w/v in TNE buffer (0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 0.001 M EDTA) using a Beckman (Beckman, Palo Alto) Sw 27 rotor for 90 minutes at 27,000 rpm at 5° C onto a 50 per cent w/v sucrose cushion. The resulting virus preparation was diluted to 1.5 ml in TE buffer (0.01 M Tris-HCl, 0.001 M EDTA), ultrasonicated (5 seconds, Bransonic waterbath 12), layered on a continuous 30 to 70 per cent w/v sucrose gradient and centrifuged for 6—8 hours (SW 41, 40,000 rpm, 5° C); 0.5 ml fractions were collected, tested for radioactivity, infectivity and by electronmicroscopy, and the peak fractions (see Fig. 4A, shaded area) pelleted (SW 41, 35,000 rpm, 60 minutes, 5° C). Non-labelled herpesvirus was purified by differential centrifugation through sucrose and CsCl gradients as reported earlier (17).

Labeling of Cells

Infected (multiplicity of infection 10 PFU/cell) or control cells were washed once with prewarmed PBS. 1.5 ml labelled medium (0.01 M HEPES buffered MEM, supplemented with 2 per cent fetal calf serum) was used for a 60 mm petri dish. When the cells were labelled with ^{3}H-glucosamine (20—50 μCi/ml), 10 mM D-fructose was used as energy source instead of glucose. Amino acid labelling was achieved with a mixture of ^{3}H-amino acids (leucine, valine and lysine, 15 μCi/ml each) or ^{35}S-methionine (10 μCi/ml) in medium, free of the corresponding components.

If not otherwise indicated the cells were labelled between 5 to 20 hours p.i. The culture fluid was carefully removed and the cells were frozen at $-70°$ C.

Indirect Immunoprecipitation Test

It followed essentially procedures outlined for other virus-cell systems (3, 4). Briefly, the cell layer (approximately 2×10^6 cells) was incubated for 20 minutes with ice cold lysis buffer (0.02 M Tris-HCl, pH 7.4, 0.05 M NaCl, 0.5 per cent Nonidet P-40), then made 0.5 per cent with sodium deoxycholate and incubated for 10 minutes. The resulting suspension was ultracentrifuged (SW 50.1, 1 hour, 30,000 rpm), and the supernatant (refered to as lysate) stored at $-70°$ C after determination of the protein concentration (16) and the TCA precipitable counts. For immunoprecipitation approx. 200 μl of lysate (60—80 μg of total protein) were maintained at 4° C overnight with 5 μl of serum in siliconized plastic tubes. Addition of the appropriate amount (see below) of anti-IgG serum (same results as with purified anti-IgG immune globulin) resulted in visible aggregation. The incubation with the second antibody was carried out for 4 hours at 4° C. Iodinated IgG served to determine the optimally precipitating

($\geqslant$95 per cent) amount of anti-IgG-serum (1). The immune complex was washed twice with ice cold lysis buffer, followed by a third wash with ice cold distilled water (each centrifugation 10 minutes, $200 \times g$).

In case of pretreatment (see results) 5 µl of serum were mixed with 200 µl of unlabelled lysate (about 160 µg of protein) or the same volume of lysis buffer and kept overnight at 4° C. Then 150 µl of labelled lysate were added, and after 4 hours 4° C the IgG was precipitated as described above. This kind of pretreatment was shown by dilution experiments to have no effect on the specificity of the reaction.

Polyacrylamide Gel Electrophoresis (PAGE)

The precipitate was dissolved in 50 µl of "sample buffer" (0.0625 M Tris-HCl, pH 6.8, 2 per cent SDS, 4 per cent 2-mercaptoethanol and 1 µl of 0.5 per cent bromphenol-blue), boiled for 5 minutes in a water bath and electrophoresed in the LAEMMLI (14) system.

8 per cent polyacrylamide gels (length: 9 cm, diameter 0.5 cm, cross linker: N, N'-methylenebisacrylamide [BIS]) with a 2.5 cm long stacking gel (2.5 per cent acrylamide) were made from a stock solution (30 per cent acrylamide, 0.8 per cent BIS). The samples were electrophoresed with a constant current of 2 mA and after the marker dye had passed the stacking gel, with 4 mA. The gels were stored frozen at $-30°$ C until cutting in 1 mm slices and preparation for liquid scintillation counting (13). Soluene 350 (Packard) served as solubilizer. A defined ^{14}C-labelled protein lysate of Semliki Forest virus infected cells or ^{14}C-labelled proteins of influenza virus infected cells, kindly supplied by G. Kaluza and H.-D. Klenk from this institute, were used for mol. wt. determinations as internal markers (see Fig. 1 A).

Chemicals and Isotopes

Acrylamide and N, N'-methylenebisacrylamide (BIS) were from Bio-Rad, München; 2-mercaptoethanol and SDS were from Serva, Heidelberg. Nonidet P-40 was a gift from Shell Chemical Co., Hamburg. All remaining chemicals, reagent grade, were from Merck, Darmstadt.

D-[6-^{3}H]-glucosamine hydrochloride (spec. act. 15,000—25,000 mCi/mmol.), L-[4.5-^{3}H]-leucine (spec. act. 59 Ci/mmol.), L-[4.5 (n)-^{3}H]-lysine monohydrochloride (spec. act. 18 Ci/mmol.), L-[2.3-^{3}H]-valine (spec. act. 16 Ci/mmol.), L-[^{35}S]-methionine (spec. act. 100,000 mCi/mmol.) and [6-^{3}H]-thymidine (spec. act. 20,000—30,000 mCi/mmol.) were obtained from Radiochemical Center, Amersham.

Results

Recognition of HSV-1 Antigens by Different Antisera

Several hyperimmune and convalescent rabbit sera against different HSV-1 strains, the titer of which was first determined by classical serological methods (Table 1), as well as a serum directed against proteins released from HSV-1 infected cells (11), were tested in the immunoprecipitation—PAGE assay system (see Materials and Methods). Figure 1 shows that all sera recognized the same antigens. The gel pattern was the same regardless of whether the host cells were of rabbit or human origin (data not shown): one major assymetrical peak (peak I) can be seen in fractions 20 to 30, corresponding to an apparent mol. wt. of 100,000. A minor second peak, not clearly resolved, is detectable in fractions 35 to 50 (peak II, apparent mol. wt. 60,000—80,000) and a third peak shows up around fraction 65 (apparent mol. wt. about 50,000). Using ^{3}H-amino acid- or ^{35}S-methionine- or ^{3}H-glucosamine- or ^{14}C-glucosamine-labelling and coprecipitation experiments (Fig. 1 D), we could show that all except one of the antigens detected

are glycosylated: one peak in the 110,000 mol. wt. region, recognized by all sera, showed up only after amino acid-labelling (Fig. 1D; 4C, D; 5B). Preimmune sera gave no specific reaction (compare profile Fig. 2E).

Table 1. *Specification of antisera directed against herpesvirus antigens*

Serum code	Immunization procedure: Virus (strain)	Immunization procedure: Application of antigen[a]	Titer[b]: CF	Titer[b]: NT	Reaction[c] in ippt. and PAGE
1. KO_2	HSV-1 (KOS) cell free	cornea scarification; [R][d] 2× boosted; i/m; s/c	16	240	+
2. H-KOS_I	HSV-1 (KOS) cell free	cornea scarification; [R]	32	300	+
3. H-KOS_{II}	HSV-1 (KOS) cell free	1× boosted; s/c; with NP-40 (0.5%) lysed, purified virus in F.a.	32	906	+
4. No. 20[e]	HSV-1 (KOS)		n.t.	265	+
5. KSW	HSV-1 (No. 1332) cell free	in F.a.; 3× boosted; [H][f] s/c	8	220	+
6. Kaplan-1[g]	HSV-1 excreted proteins		n.t.	125	+
7. L 8[h]	BHM (TVA) purified	in F.a.; 3× boosted; [H] s/c	16	70	+
8. LK_I	IBR (RF) purified	in F.a.; 2× boosted; [H] s/c; 1× boosted; with $Al_2(SO_4)_3$ treated virus; i/p	<4	<5	−
9. No. A-356[i]	PsR (DEK) purified	UV inactivated, in [H] F.a.; 2× boosted; s/c	n.t.	<5	−
10. $A1_3$	PsR infected cells, glutaraldehyd fixed	in F.a.; 3× boosted; [H] s/c	n.t.	<5	−
11. SWA_3[h]	BHM (TVA) cell free	skin scarification; [H] s/c; i/v; 3× boosted; s/c; i/v	32	64	+

Sera 1 to 10 were prepared in rabbits, serum 11 was from a cow

[a] If Freund's adjuvant (F.a.) was used, the first injection was done with complete F.a. and boosters with incomplete F.a.

[b] Reciprocal titers in complement fixation (CF)- and neutralization tests (NT; 80 per cent plaque reduction), when tested against HSV-1 antigen or cellfree virus

[c] (+), significant counts over background were detected in immuno-precipitation (ippt.) of peak I and II; (−), no significant reaction

[d] [R], convalescent serum

[e] Serum kindly supplied by M. Benyesh-Melnick, Houston, in 1971

[f] [H], hyperimmune serum

[g] Serum kindly supplied by A. S. Kaplan, Nashville

[h] Further details given by Sterz *et al.* (35)

[i] Serum prepared by R. Floyd and H. Ludwig at Baylor Coll. Med., Houston, in 1971; 50 per cent plaque reduction titer against PsR virus 1:320

i/m, intramuscular; s/c, subcutaneous; i/p, intraperitoneal; i/v, intraveneous

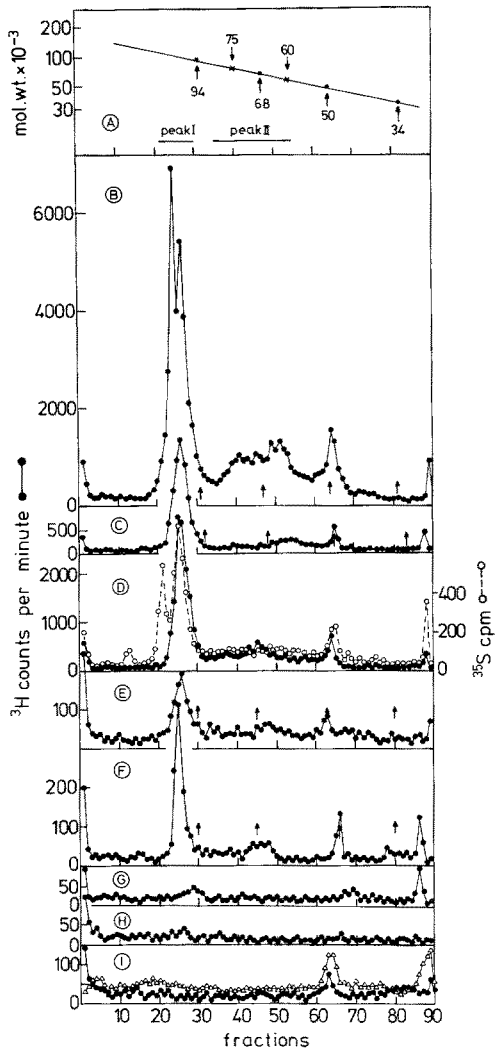

Fig. 1. PAGE analysis of HSV-1 antigens precipitated by rabbit antisera (compare also Table 1).

^{3}H-glucosamine labelled (5—20 hours p.i.) antigens from lysates of strain KOS infected RK cells (spec. act. 2.3×10^3 cpm/μg protein) were reacted with: KSW, hyperimmune serum against a fresh HSV-1 isolate (B); No. 20, convalescent serum against strain KOS (C); hyperimmune serum against excreted proteins (E), (11); L 8, hyperimmune serum against purified BHM virus (F); LK_I, hyperimmune serum against purified IBR virus (G); A-356, hyperimmune serum against purified PsR virus (H) · (D), shows the coprecipitation of ^{3}H-glucosamine and ^{35}S-methionine labelled antigens of infected cells by H-KOS_{II}, a convalescent serum. In (I) ^{3}H-glucosamine (•———•) or ^{3}H-amino acids (△———△) labelled uninfected RK cell-lysates were reacted with KO_2, a HSV-1 specific convalescent serum, and analysed in parallel gels. (A), calibration curve for mol. wt. determinations, using defined Semliki Forest virus (↑) and influenza virus (↓) specific proteins as internal markers

Since the complexity of the humoral immune response is well known (6, 23), we compared the antigens detected by experimentally made antisera with the ones precipitated by antibodies from natural HSV infection.

Figure 2 shows the results obtained with lysates of strains KOS or H_4, respectively, infected rabbit cells reacted with human serum pools. A similar glycoprotein pattern was obtained when a fresh isolate (NIC) was used for infection of the cells (data not shown). As with the rabbit sera the glycoproteins with an apparent mol. wt. of 100,000 are most prominent, the 50,000 mol. wt. peak being also visible in about identical proportion. The glycoproteins in the 60,000 to 80,000 mol. wt. region are now more or less distinct (see below). Sera free of neutralizing antibodies gave no specific reaction (see Fig. 2E).

Experiments with 11 individual sera and 3 serum pools and a variety of lysates (some representative results are given in Table 2) led to the conclusion that there is no evident correlation between the titers in CF- and neutralization-tests and the amount of immunoprecipitated radioactivity in the 2 major glycoprotein regions (peak I and II) on the one hand, and between the peaks I and II themselves on the other hand. The latter proportion seems to depend on the individual serum. That antibody is indeed the limiting factor in our test system, is demonstrated by the results shown in Figure 2A.

Table 2. *Comparison of complement fixation (CF)- and neutralization (NT) titers of sera and CSF with the amount of radioactivity immuno-precipitated (peak I and peak II) from HSV-1, strain KOS, infected cells*

Lysate	Code	CF[c]	NT[c]	Peak I (cpm)	Peak II (cpm)	Peak I / Peak II
KOS-RK_{13}[a]	UHL[d]	32	380	5,990	1,940	3.1
KOS-RK_{13}	ELL[d]	32	640	6,110	3,360	1.8
KOS-RK_{13}	ROS pool[d]	32	200	6,660	2,950	2.3
KOS-RK_{13}	CSF-MÖB[e]	16	30	9,210	3,930	2.3
KOS-RK_{13}	KO_2[f]	16	240	11,100	2,500	4.4
KOS-RK[b]	GP pool[d]	64	480	9,090	5,680	1.6
KOS-RK	URS pool[d]	32	300	8,670	2,730	3.2
KOS-RK	JAK[d]	256	710	7,300	4,660	1.6
KOS-RK	CSF-MÖB[e]	16	30	7,125	4,400	1.6
KOS-RK	H-KOS_I[f]	32	300	10,180	2,290	4.1
KOS-RK	H-KOS_{II}[f]	32	906	7,370	1,800	4.1
KOS-RK	KSW[f]	8	220	26,700	11,600	2.3
KOS-RK	No. 20[f]	n.t.	265	9,800	1,600	6.1
KOS-RK	Kaplan-1[f]	n.t.	125	730	230	3.2

Spec. act. of ^{3}H-glucosamine labelled proteins

[a] 3.6×10^3 cpm/µg

[b] 1.0×10^4 cpm/µg

[c] Reciprocal titers in CF and NT (80 per cent plaque reduction)

[d] Human sera

[e] Human cerebrospinal fluid

[f] Rabbit sera

For further details see Materials and Methods and Table 1

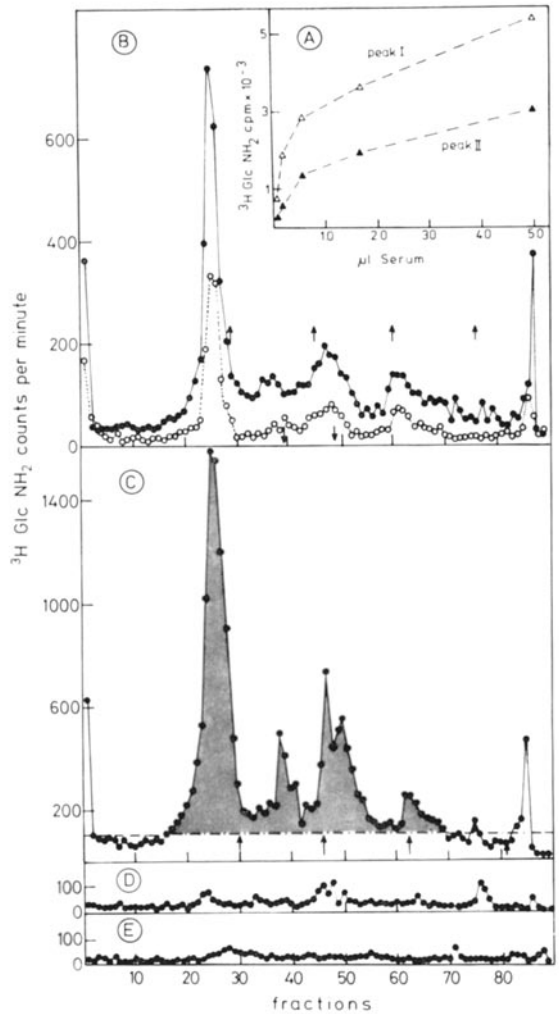

Fig. 2. PAGE analysis of glycoproteins from HSV-1 infected cells after immunoprecipitation by natural antibodies.
(B) Lysate from strain KOS (•————•) infected RK_{13} cells (labelled from 5—20 hours p.i., spec. act. 3.6×10^3 cpm/µg protein) precipitated with human serum (ROS pool). On a parallel gel strain H_4 (o— — —o) infected RK cells (labelled from 6—25 hours p.i., spec. act. 1.1×10^3 cpm/µg protein) reacted with human serum (GP pool). (A) Titration of a human pool serum (GP pool). This serum was diluted in a human serum containing no HSV antibodies (RR-1 serum) and then 5 µl of each dilution were reacted with the same amount of an infected cell lysate (strain KOS). The immune precipitate was electrophoresed. The counts obtained with the negative serum (RR-1) were subtracted for quantitation of peak I and II glycoproteins. (C) Antigens detected by 20 µl of CSF (MÖB) and (D) by 20 µl of serologically negative CSF; 5 µl of negative human serum (RR-1) had been added as carrier, for calculation of the peak ratios (see Table 2) the background counts (non-shaded area) were subtracted. (E) Analysis of the precipitate performed with the negative human serum alone

In view of the poverty of the data about the immune response induced in the brain by herpesvirus encephalitides and the possibility of selective antibody production as observed in the course of other neurotropic virus diseases (25), we tested human CSFs containing antibodies in the following way: because only limited amounts were available a human serum negative in neutralization-, CF- and immunoprecipitation tests against HSV-1 antigens (Fig. 2E) was used as carrier in the precipitation assay (see legend Fig. 2). The glycoproteins detected in the gel are apparently the same as the ones found with sera. The much better resolution seen with the CSFs (Fig. 2C) allowed, however, apparent mol. wt. determinations of 100, 80, 75, 67, 64, and 50×10^3. CSFs from patients with non-herpetic encephalitides gave no significant reaction (Fig. 2D). The better separation achieved by the CSFs compared to patient- or animal sera might be due to the relative prevalence of virus specific immunoglobulins in CSF.

Specificity Controls

Antisera against other herpesviruses like IBR or PsR virus or against PsR virus infected cells (see Table 1), which do not neutralize HSV-1, showed no precipitation of HSV-1 specific glycoproteins (Fig. 1G, H). However, rabbit serum directed against purified BHM virus (Fig. 1F) and bovine convalescent sera taken after natural or artificial BHM virus infection specifically precipitated peak I from HSV-1 infected cell lysates (data not shown). These sera were known to cross-react with HSV-1 antigens (35). Considering the possibility that the precipitated material could include cellular proteins, we incubated HSV-1 specific sera with labelled uninfected cells. A small peak in the 50,000 mol. wt. region was visible (Fig. 1I). On the other hand, pretreatment of HSV antibody positive rabbit and human sera and human CSFs, as well as antiserum against BHM virus with lysates of uninfected unlabelled cells (see Materials and Methods) did not influence the gel pattern except in lowering the background 3 to 4 fold and diminishing the 50,000 mol. wt. glycoprotein (Fig. 3A).

Obviously this peak represents a cellular protein which is either recognized by antibodies or non-specifically coprecipitated. Such proteins have been reported with this technique also in other virus-cell systems (3, 5).

By the use of lysates of strains KOS, XIII or H_4 infected unlabelled rabbit or human cells and of purified KOS virus for pre-treatment of the sera we could completely eliminate their ability to recognize labelled strain KOS specific antigens (Fig. 3B, C, D). These results suggest that the virus specific glycoproteins of these three HSV-1 strains detected by antisera have common antigenic sites. This would be in line with reported neutralization experiments (27).

As a further control we pre-treated the sera with lysates of unlabelled PsR- or IBR virus infected RK cells or lysates from purified PsR Virus. The heterologous viral antigens did not absorb out antibodies directed against HSV-1 specific glycoproteins (Fig. 3A). This is of special interest since PsR virus and HSV-1 share about 10 per cent genetic information (19) and show restricted antigenic relationship (8).

The Antigens of Purified HSV-1

Strain KOS virus was purified from the supernatant without pelleting to avoid virus aggregation and eventual contamination with cellular material

(Fig. 4A) as detailed in Materials and Methods. Immunoprecipitation of lysed glucosamine or amino acid labelled virus with rabbit serum (Fig. 4B) or human serum (GP pool, Fig. 4C) resulted in a glycoprotein gel pattern almost superimposable upon the one emerging when untreated whole virus was electrophoresed in parallel. The unglycosylated protein with an apparent mol. wt. of 110,000 (Fig. 4C), which was reproducibly obtained from purified virus, was not investigated further.

PAGE analysis of purified virus and differently labelled immunoprecipitates of lysates from infected cells in the same gel (Fig. 4D) demonstrates that the difference between the virus- and the lysate-originating patterns concerns the smaller mol. wt. region ($\leqslant$50,000), where non-glycosylated proteins are detected.

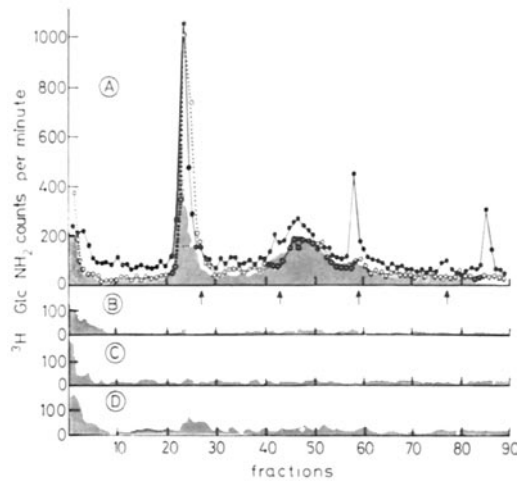

Fig. 3. Effect of antiserum pre-treatment on precipitation of HSV-1 (strain KOS) antigens from infected cells.
Human serum (GP pool), pre-treated as described in Materials and Methods, with lysis buffer (•———•), lysates of purified PsR virus (o — — — o, 40 μg of total protein) and of uninfected RK cells (shaded profile), reacted with labelled antigens (cells labelled from 5—20 hours p.i.) and electrophoresed on separate gels (A). Pre-treatment of the same serum with lysates of HSV-1, strain XIII, infected RK cells (B), strain H_4 infected human embryonic oligodendroglia cells (C) and purified HSV-1, strain KOS (40 μg of total protein) (D), prior to immuno-precipitation

Appearance of the Virus Specific Antigens in the Cell and on the Cell Surface

In order to obtain optimal yields of virus-specific proteins and avoid cellular protein synthesis we usually labelled the infected cells from 5—20 hours p.i. The appearance of antigens after infection was investigated by labelling the cells with glucosamine or amino acids for 2 hours intervals throughout the replication cycle. Beginning with the 2—4 hours p.i. labelling period the proteins in peaks I and II can be recognized (Fig. 5). The proportion of peaks I and II remains roughly the same in cells pulse labelled for the 2 hours intervals compared to those labelled from 8—23 hours p.i. After detecting these antigenic components early in the infection cycle we examined the question whether and when they would appear

on the cell surface. For this reason immunoprecipitation tests were performed with sera which had been absorbed with intact infected cells (see Materials and Methods), harvested every 2 hours between 0—8 and at 23 hours p.i. (a growth curve was done using virus from parallel cultures to make sure that the cells propagated

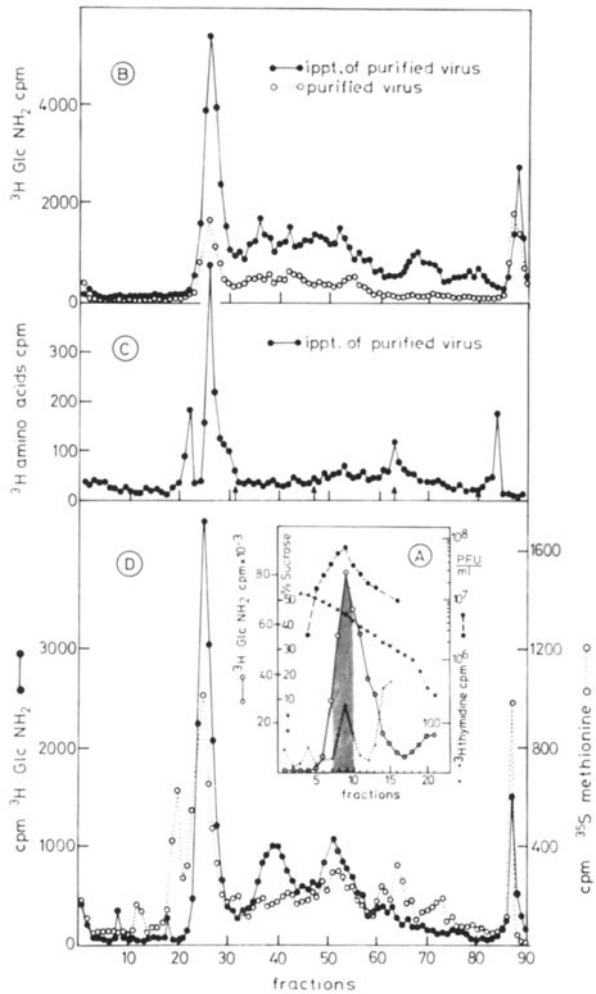

Fig. 4. Analysis of immuno-precipitated antigens from purified HSV-1 (strain KOS) by PAGE.

Infected human embryonic oligodendroglia cells were labelled with ^{3}H-glucosamine or ^{3}H-amino acids and infected RK cells with ^{3}H-thymidine (4—30 hours p.i.). Supernatant virus was purified as described in Materials and Methods (A). The marked virus fractions (shaded area) were analysed in (B) and (D). ^{3}H-thymidine labelled virus was run in a parallel gradient. (B), lysed virus was immuno-precipitated (ippt.) with rabbit serum H-KOS$_{II}$ (•———•) and electrophoresed on a parallel gel to purified virus alone (o — — —o). (C), antigens from a lysate of purified virus (^{3}H-amino acids labelled) precipitated with human serum (GP pool). (D), precipitate of ^{35}S-methionine labelled infected cell antigens and human serum (GP pool) mixed with virus and coelectrophoresed

the virus in a single cycle replication, see Fig. 6). The peak I glycoproteins were slightly reduced only if the sera had been absorbed to cells infected for 8 and 23 hours, respectively. Antibodies recognizing the peak II region, however, disappeared gradually starting from about 2 hours post infection when no infectious virus could be detected. 70 per cent of the antibodies were absorbed by cells taken at 8 hours p.i. and 90 per cent by cells taken at 23 hours p.i. (Fig. 6 and inset A). We cannot exclude that at later times (from 6 hours p.i. on) virus adhering to cells also participates in antibody absorption.

Aliquots of the absorbed sera were tested for their virus neutralizing capacity in parallel to the immunoprecipitation assay. A clear reduction in titer shows up by the use of sera treated with cells harvested at 4 hours p.i. The neutralization titer continues to decrease down to 20 or 10 per cent of the initial titer after absorption with cells harvested 8 or 23 hours p.i. (Fig. 6 and inset B).

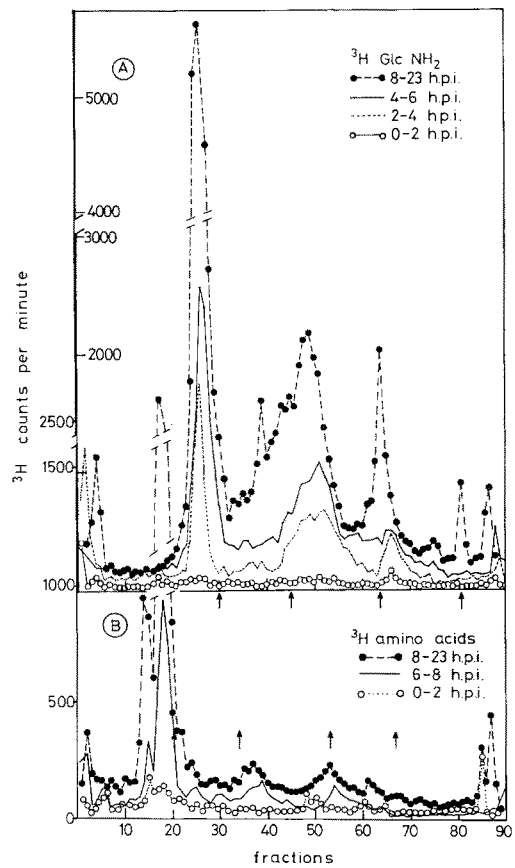

Fig. 5. Appearance of HSV-1 antigens in infected cells. Equal amounts of protein (80 μg) from lysates of 2×10^6 (strain KOS) infected RK cells (pulse labelled as indicated) were reacted with a human serum (GP pool). The precipitates were analysed on parallel gels [8 per cent polyacrylamide in (A) and 10 per cent in (B), respectively]. The data of cells labelled from 6—8 hours p.i. (A) and from 2—4 and 4—6 hours p.i. (B) are not shown

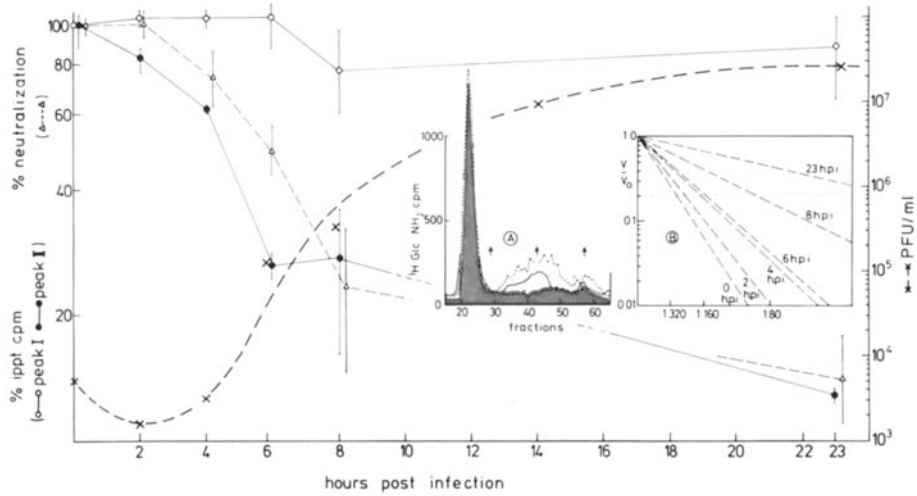

Fig. 6. Appearance of HSV-1 antigens on the cell surface and their accessability to neutralizing antibodies. Strain KOS infected RK cells were harvested at the indicated times p.i. and used for absorption of aliquot samples of a human serum (GP pool), as described in Materials and Methods. 10 µl of each absorbed serum were pre-treated with a lysate of uninfected unlabelled RK cells (see Materials and Methods) and reacted with a lysate from KOS infected RK cells, labelled from 5—23 hours p.i. with ^{3}H-glucosamine. The results of 2 experiments are averaged. The cells used for absorption came from the experiments described in Fig. 5. The counts in peak I and peak II, respectively, were quantitated after correction for background, and expressed as percent counts immuno-precipitated (ippt.). The data obtained with cells harvested 0 hour p.i. are set 100 per cent. Inset (A) shows PAGE profiles (experiment 1) of precipitates obtained with serum samples, which had been absorbed with cells harvested at 0 (· · ·), 6 (———) and 23 (○———○, shaded profile) hours p.i. The arrows indicate marker proteins (mol. wt. 94, 68, 50×10^3). In inset (B) the virus neutralization obtained with the absorbed sera is plotted as fractional reduction of plaque counts V/V_0 against serum dilution (V is the number of plaques in serum treated virus samples and V_0 [about 200 plaques] in control samples). The dotted line in inset (B) marks the 80 per cent plaque reduction titer, which is expressed in the figure as percent neutralization (△— — —△). The growth curve was done with intracellular virus from cultures of experiment 1

Discussion

We have characterized antigens from HSV-1 infected cells and from the virion which are recognized by human and rabbit HSV specific antibodies and have tried to elucidate their possible role in virus neutralization. PAGE analysis revealed that antisera detected two major glycoprotein groups: one with an apparent mol. wt. of 100,000 (peak I) and a second of about 60,000—80,000 (peak II). Their ratio seems to depend on the individual serum. It is of interest that cerebrospinal fluids of patients with herpes encephalitis are able to immunoprecipitate distinct glycoprotein species in the broad peak II region, however, antibodies present in these fluids produce the same overall pattern. A further glycoprotein peak (apparent mol. wt. 50,000) does not seem to be virus-related, since it could also be precipitated from lysates of uninfected cells and was dimin-

ished by pre-treatment experiments (see Results). Fleissner and Tress (3) have discussed the complications in identifying viral and cellular proteins with this type of analysis. The only non-glycosylated antigen detectable (apparent mol. wt. 110,000) seems to correspond to the major nucleocapsid protein which has been investigated by others (29, 34).

We are aware of the possibility that, under the conditions of lysis used, not all virion proteins might have been solubilized. Nonidet-P40 and deoxycholate should bring membrane proteins into solution, but nucleocapsid polypeptides might be partially lost. This means that our results cannot be compared directly with other antigen-assay systems (8, 31, 36).

The comparison of precipitated antigens from lysates of infected cells or of purified virus with purified untreated virus shows that only structural components of the virion have been found and no glycoproteins besides the ones immunoprecipitated could be detected in the virion.

The time-course experiments studying the appearance of the two major antigenic components (peaks I and II) show that these glycoproteins are already present early in the virus replication cycle and that mainly peak II proteins become accessible to antibodies on the cell surface. The inability to neutralize HSV-1 and to precipitate peak II glycoproteins with sera which were absorbed with cells harvested late after infection strongly suggests that peak II antigens are involved in the neutralization process and are more accessible to antibodies than the 100,000 mol. wt. glycoproteins. Our data are compatible with the assumption that the antigenic sites of the peak I glycoproteins are less exposed on the cell membrane, than the peak II glycoproteins. The accessibility of the peak II antigens on the cell surface suggests that they represent antigens detected already by other methods (7, 24). The kinetics of appearance of peak II antigens correlates well with the increase and levelling off in direct binding of radioactively labelled HSV-1 specific rabbit IgG (26) or indirect binding of ferritin tagged anti human IgG to HSV infected cells (32). Because of the obvious exposure of peak II glycoproteins, it is possible that they also play a significant role in cellular immunity to HSV infection (15).

The participation of the 60,000—80,000 mol. wt. glycoproteins in neutralization does not necessarily contradict the reports that neutralizing antibodies have been raised against an isolated glycoprotein complex, which probably corresponds to our peak I antigens (22, 29) and seems to represent the major envelope protein (34). In this context it should be mentioned that antiserum against excreted HSV-1 specific glycoproteins (11), which has neutralizing activity, in our hands recognized mainly peak I antigens. We also have preliminary evidence that the glycoproteins with an apparent mol. wt. of 100,000 participate in virus neutralization, since the genetically and immunologically related herpes simplex virus types 1 and 2 and BHM virus (2, 19, 35) share an antigenic site on glycoproteins appearing in the peak I region (Ludwig *et al.*, in preparation). It might well be that the presence of complement plays a decisive role in the neutralization process in which one or the other group of glycoproteins (peak I or II) is involved.

Our data demonstrate that two major groups of glycoprotein species (peaks I and II) give rise to antibodies in herpes simplex virus infection. They confirm and extend the results of Spear (33), who reported first that a limited number of

virus-specified proteins are immunoprecipitated by rabbit sera, although our peak II glycoproteins, which appear to correspond to viral proteins 12—14 (34), seem not to be detected under different experimental conditions (33). There is a close similarity in the gel profiles of glycoproteins immuno-precipitated with our sera and those studied with immuno-absorband columns made with rabbit antibodies (30). However, a definite answer concerning the functional role of these glycoprotein species still awaits further biochemical and immunological characterization.

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