Isolation of a Herpes Simplex Virus-Specific Antigenic Fraction Which Stimulates the Production of Neutralizing Antibody

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Infection of mammalian cells with herpes simplex virus (HSV) results in the production of a number of virus-induced soluble antigens. Immunodiffusion analyses of the soluble antigen mixture (SAM) obtained from HSV-infected KB or BHK cells revealed at least six well-defined immunoprecipitin bands. Calcium phosphate chromatography (Brushite) was employed to separate one immunoprecipitin (designated CP-1) from the remaining viral and host antigens. We conclude that CP-1 is a viral-specific antigen because (i) specific antiserum, which had been repeatedly absorbed with uninfected cell extracts or serum components, still retained the capacity to react in gel diffusion with CP-1 antigen; (ii) anti-CP-1 serum reacted in gel diffusion with SAM, yielding one precipitin band in identity with the band formed against human gamma globulin; (iii) the CP-1 fraction stimulated the production of HSV-neutralizing antibody of high capacity. The last observation suggests that fraction CP-1 contains a biologically active structural component of the virus which is associated with the envelope. The CP-1 immunoprecipitin was separated from SAM by an alternative method by using a cyanogen bromide-linked immunosorbent prepared from anti-CP-1 gamma globulin. The observation that the CP-1 antigen isolated from the immunosorbent effectively blocked serum-neutralizing activity provided further evidence that neutralizing antibody was directed against CP-1. Acrylamide gel electrophoresis and immunological experiments suggest that the CP-1 antigen is in part a glycoprotein. The finding that CP-1 contains only one antigenic component of the virus will permit future biological studies to be made with a monoprecipitin antiserum. In addition, the techniques described in this paper represent initial steps in the purification of HSV antigens.

The most direct approach to the study of herpes simplex virus (HSV)-specific proteins would be to separate and examine those antigenic moieties which are actually incorporated into the mature virus particle. There are two important prerequisites for such studies: (i) preparation of highly purified virus pools in sufficient quantities which contain no contaminating host cell proteins; and (ii) a procedure that completely disrupts the virus particles and frees the structural components without destroying their antigenic properties. As yet, neither of these prerequisites has been met satisfactorily.

Most recent investigations into the nature of HSV proteins have concentrated upon analysis of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis patterns of infected cell extracts and purified virus (12, 14, 23). These experiments have provided much information about viral proteins, including estimates of the number of viral polypeptides and their relative molecular weights, and the position of each in the virus particle, as well as time of synthesis. However, because of the denaturing action of SDS and urea, it is difficult to assign a specific biological activity to individual polypeptides. Furthermore, the effectiveness of these procedures relies heavily upon obtaining purified viral preparations (14, 23).

An alternative approach utilizes immunological methods to study the soluble virus-specific antigens which are found in mammalian cells after viral infection (4, 35, 36). Some of these antigens represent virus structural components produced in excess, and others are probably virus-induced enzymes. The immunological procedures presumably do not cause a loss of biological activity. Although attempts have been made to separate
and characterize HSV-soluble antigens, no simple and reproducible method has been described (27, 28, 30, 32).

This report describes a method for the isolation of an HSV-specific immunoprecipitin from a mixture of other viral-induced antigens. Our studies suggest that this antigenic moiety is a structural component of the viral envelope.

MATERIALS AND METHODS

Cell cultures. Monolayer cultures of KB cells were propagated in 32-oz (0.95-liter) bottles containing Eagle minimal essential medium (MEM) supplemented with 10% calf serum. BHK cells were propagated in 32-oz bottles or roller bottles by using MEM supplemented with 5% fetal calf serum.

Virus and virus titration. HSV (strain HF) was used throughout. This strain elicits syncytium formation in BHK cells and rounded cells in KB cultures. The preparation of high-titered virus stocks and plaque titrations was performed as described previously (3), but omitting the agar overlay and with 5% fetal calf MEM as a maintenance medium.

Soluble antigen preparations. Monolayer cultures of KB or BHK cells were infected at an input multiplicity of 5 to 10 plaque-forming units (PFU) per cell. The cells were harvested after 18 to 24 hr, washed three times with phosphate-buffered saline (PBS), pH 7.2, and concentrated by centrifugation (10 min at 800 × g). The cell pellet was suspended in PBS to a final concentration of 2 × 10^7 cells per ml and treated for 45 sec in a 60-w MSE Sonifier (Measuring and Scientific Equipment Ltd., London). Such preparations were centrifuged for 2 hr at 100,000 × g, after which the supernatant fluids were collected, dialyzed overnight against 0.01 M phosphate buffer (pH 7.2), and finally concentrated (usually three- to fourfold) in a Diaflo ultracentrifugation chamber with an XM-50 membrane (Amicon Corp.). Protein concentration was determined by the method of Lowry et al. (9) by using crystalline bovine serum albumin as a standard. This crude material was termed the soluble antigen mixture (SAM). Prior to preparation of antisera, antigen preparations were rendered free from infectious virus by the following procedure: HSV-SAM was centrifuged at 100,000 × g for 2 hr at 4 C. The top two-thirds of the supernatant fluid was aspirated and centrifuged once more at 100,000 × g. This procedure was repeated for a third time. The efficacy of this procedure for the removal of infectious virus was monitored by virus titration. Even after several blind passages through BHK cell monolayers, the final antigen preparation failed to produce plaques.

Preparation of antisera. Antisera directed against viral-induced soluble antigen(s) were prepared by immunizing rabbits with a series of three intramuscular injections of the appropriate antigen suspended in Freund complete adjuvant. Ten days after the last injection, the rabbits were boosted intravenously with adjuvant-free antigen. All antisera were absorbed with extracts of uninfected KB or BHK cells and cell serum to remove antibody directed against nonviral components. Serum was considered absorbed when it did not react by immunodiffusion with uninfected cell extracts at a concentration of 8 mg of protein per ml. This concentration of protein was 2 to 30 times greater than in the viral antigenic fractions used for immunodiffusion. Human gamma globulin was obtained commercially (Dow Chemical Co.).

Calcium phosphate chromatography. The Brushite form of calcium phosphate was prepared according to the method of Tiselius et al. (25), and absorption chromatography was carried out by the method of Tavenre et al. (24). Brushite is relatively unstable and was prepared 1 day prior to chromatography and then was washed overnight with 0.001 M potassium phosphate buffer at pH 7.0. Samples were dialyzed overnight against 0.005 M phosphate, pH 7.0, and added to the columns. All experiments were done at 4 C.

Virus neutralization test. The plaque reduction technique was used in the same manner as previously described (4). The greatest dilution of serum causing a 50% reduction of the plaque count (on BHK cell monolayers) as compared with virus-preimmune serum mixtures was selected as the neutralizing titer.

Serum blocking activity. The technique used was essentially that of Zwartouw and Westwood (37). Viral-specific antisera were employed at a dilution capable of reducing the number of HSV PFU by 50%. Extracts prepared from infected or uninfected cells, at various protein concentrations, were incubated with the antisera for 1 hr at 37 C. A known quantity of HSV was then added to the antigen-antibody mixture, incubated for 1 hr, and plated on BHK monolayers. The surviving virus was titrated as described previously (4). Control mixtures consisted of: (i) HSV incubated with diluent and preimmune rabbit serum; and (ii) HSV incubated with viral-specific antiserum at a dilution allowing 50% survival. The capacity of an antigenic preparation to block the virus-neutralizing ability of an antiserum was calculated according to the following equation: (b - a) / (b - c) × 100 = percent of serum blocking capacity.

Immunological techniques. Gel diffusion was performed on Plexiglas templates by the method of Crowle (5) with 0.8% agar no. 2 (Colab) in 0.05 m Veronal buffer, pH 8.6. Immunoelectrophoresis was carried out by the method of Scheiddegger (22). The immunospecific absorbent (immunosorbert) was prepared by mixing anti-CP-1 gamma globulin, fractioned from serum according to Montgomery et al. (10), with cyanogen bromide-activated Sepharose 2B (Pharmacia (1, 6). Briefly, Sepharose 2B (14 ml settled volume) was mixed with an equal volume of cyanogen bromide at 25 mg/ml. The pH was immediately adjusted to 11, with maintained at, 11 to 11.5 with 2 N NaOH (ca. 8 ml added in drops). When the reaction had ended (as judged by the stabilization of pH at 11), the mixture was washed with 1 liter of cold water followed by 1 liter of 0.05 m cold borate-buffered saline, pH 8.4. The drained, moist, activated Sepharose was scraped into a beaker, 70 mg of absorbed viral-specific rabbit gamma globulin was added, and the mixture was stirred at 4 C for 48 hr. At the end of this time, the reaction was terminated by the addition of 0.05 m ethanolamine. The immunosorbert was washed with PBS until no more ultraviolet-absorbing material
appeared in the wash. Approximately 90% of the input protein was bound to the activated Sepharose.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the technique developed by Maizel (20). Samples containing 50 to 200 μg of protein were treated with 0.5% SDS, 0.5 M urea, and 0.1% mercaptoethanol. The preparation was heated at 37 C for 1 hr and cooled to room temperature. Sucrose was added to a final concentration of 10%, and bromophenol blue was added as a tracing dye. Samples were layered on top of acrylamide gels prepared in glass tubes (0.6 cm in diameter and 10 cm in length). The gels consisted of 6% acrylamide, 0.16% bis-acrylamide, 0.5 M urea, 0.1% SDS, 0.1 M phosphate (pH 7.2), 0.03% N, N', N'-tetramethylethylenediamine (TEMED), and 0.01% ammonium persulfate. Gels were run in the electrophoresis chamber by using 0.1 M phosphate buffer, pH 7.2, containing 0.1% SDS with a current of 10 ma per gel for 4 hr. After each run, the gels were removed from the tubes, fixed overnight in 10% trichloroacetic acid, and fractionated (about 1 mm per fraction) in a gel fractionator (Savant Instruments, Inc.). Excess water in the fractions was removed by heating for approximately 7 hr at 56 C, and the gels were suspended in 0.5 ml of 30% H2O2 and incubated overnight at 56 C. A 10-ml amount of Aquasol (New England Nuclear Corp.) scintillation fluid was added to the cooled gels, and the radioactivity was determined in an Intertechnique liquid scintillation counter. Molecular-weight estimations of the polypeptides were performed by the methods of Shapiro et al. (21) and Weber and Osborn (34). Gels were standardized with the following purified proteins: cytochrome c (12,000 daltons), chymotrypsin (25,000 daltons), ovalbumin (43,000 daltons), aldolase (40,000 daltons), catalase (60,000 daltons), bovine serum albumin (67,000 daltons), and rabbit gamma globulin (150,000 daltons).

Labeling of cells. Isotopically labeled SAM was obtained from uninfected or infected BHK cells by incubating the culture in medium containing 3H-glucosamine (1.0 μCi/ml, specific activity of 2 Ci/m mole; New England Nuclear Corp.), 3H-glucosamine (1.0 μCi/ml, specific activity of 2 Ci/m mole; New England Nuclear Corp.), 3H-galactosamine (1.0 μCi/ml, specific activity of 2 Ci/m mole; New England Nuclear Corp.), 3H-galactose (1.0 μCi/ml, specific activity of 2 Ci/m mole; New England Nuclear Corp.), or 3H-leucine (2 μCi/ml, specific activity of 2 Ci/m mole; New England Nuclear Corp.). The radioactive label was added to the culture 3 hr after infection. The cells were harvested at 18 hr after infection, and HSV-SAM was prepared as described above.

RESULTS

Characterization of HSV-soluble antigen mixture by gel diffusion analysis. A complex pattern of immunoprecipitin bands was obtained (Fig. 1) when SAM prepared in BHK cells was tested with antibody prepared against this antigen preparation. An identical pattern was observed when the same antibody was tested against HSV-SAM prepared in KB cells. Furthermore, the absorbed serum did not react with components in uninfected KB or BHK cell extracts. We estimate that at least six viral-induced antigens were synthesized after infection regardless of the host cell employed for preparation of SAM.

Separation of a single viral-induced immunoprecipitin from HSV-SAM by calcium phosphate (Brushite) chromatography. Figure 2 shows the elution profile of HSV-SAM proteins removed from a Brushite column in a stepwise fashion with increasing concentrations of phosphate buffer. The eluate was pooled in four fractions as indicated in Fig. 2, dialyzed against 0.01 M phosphate buffer, pH 7.2, and concentrated. Examination of the individually pooled column fractions by gel diffusion, with anti-SAM sera, showed partial separation of precipitating antigens.

The antigenic content of each fraction was evaluated further by preparing specific antisera against each pooled fraction (anti-CP-1, 2, 3, and 4). Figure 3a (and see diagram in Fig. 3b) indicates that anti-CP-1 serum reacted with HSV-SAM to yield one immunoprecipitin band. The specificity of the antiserum suggested that fraction CP-1 contained only one viral-induced immunoprecipitin. This interpretation was supported by the reciprocal experiment in which
fraction CP-1 was reacted against anti-SAM serum, anti-CP-1 serum, and human gamma globulin. Only one band of identity was observed with the three sera (Fig. 3c). Furthermore, immunoelectrophoresis of the CP-1 fraction showed only one band migrating toward the cathode. These results are consistent with the idea that we have separated one viral-induced immunoprecipitin and have prepared a specific antiserum to this component.

Pertinent information concerning Brushite chromatography. Prior to chromatography, an extract from uninfected BHK cells that had been labeled with 14C-leucine for 18 hr (106 counts/min) was added to the unlabeled HSV-SAM to identify normal BHK proteins. In the experiment shown in Fig. 2, total recovery of Folin protein from the column was 75%, and total recovery of 14C-protein was 78%. Fraction CP-1 constituted 15% of the total recovered protein (Folin) but only 4% of the 14C-protein. The pooled eluates from CP-2 (CP-3, which was similar to CP-4, is not shown) and CP-4 also contained detectable amounts of antigen CP-1 (Fig. 3a) as well as other precipitin bands. It is apparent that CP-1 possessed a spectrum of affinities for Brushite. Indeed, a significant amount of this immunoprecipitin, as judged by gel diffusion, was eluted in the wash and at low phosphate concentrations. Fraction CP-1 also contained at least two BHK cell-specific immunoprecipitin bands which were observed by reacting unabsorbed anti-CP-1 serum with extracts from uninfected BHK cells. Thus, calcium phosphate fractionation yielded only a partial purification of this viral component. Nevertheless, the useful feature of this procedure was that a single viral-induced immunoprecipitin could be easily and reproducibly separated from the remaining viral antigens, as well as the majority of host proteins.

Neutralization of HSV infectivity by antiserum

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**Fig. 2.** Elution profile of proteins removed from a Brushite column in a stepwise fashion with increasing concentrations of potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The column was equilibrated overnight with 0.001 M phosphate and checked for channeling by employing bromocresol purple. For this experiment, 70 mg of HSV-SAM was applied to the column in 10 ml of 0.005 M phosphate buffer. The column size was 30 by 2.5 cm, and the flow rate was 50 ml/hr.

**Fig. 3.** Gel diffusion analysis of the individually pooled column fractions obtained by calcium phosphate chromatography of HSV-SAM. a, Center well: HSV-SAM (protein concentration, 5.7 mg/ml). Peripheral wells: 1, anti-CP-1 serum; 2, anti-CP-2 serum; 3, anti-CP-4 serum; 4, anti-SAM serum. b, Diagrammatic representation of Fig. 3a. c, Center well: CP-1 antigen (protein concentration 0.3 mg/ml) Peripheral wells: 1, anti-CP-1 serum; 2, anti-SAM serum; 3, empty well; 4, human gamma globulin (diluted threefold with saline).
specific for CP-1. Table 1 illustrates that rabbit antiserum against virus-free HSV-SAM proved highly efficient in neutralizing HSV infectivity. The antiserum neutralized virus infectivity by 50% at a dilution of approximately 1 to 800. Rabbit antiserum prepared against virus-free CP-1 fraction exhibited an equivalent virus-neutralizing capacity. Human gamma globulin also neutralized virus infectivity, but to a lesser extent. We conclude that the viral-specific immunoprecipitin contained in fraction CP-1 is involved in the stimulation of neutralizing antibody. Furthermore, this biological function suggests that fraction CP-1 contains a structural component of the virus associated with the enveloped surface.

Isolation of CP-1 immunoprecipitin from HSV-SAM employing an immunosorbent. The next series of experiments was designed to explore further the idea that the CP-1 immunoprecipitin was responsible for the production of virus-neutralizing antibody. This concept would be reinforced if we could show that the CP-1 immunoprecipitin retained the same biological activity after being separated from the other viral antigens by a different method. The method we chose involved the use of the specific antiserum to CP-1 (gamma globulin fraction) for preparation of a cyanogen bromide-linked immunosorbent (1, 6). The CP-1 antigen was then selectively separated from the mixture of viral antigens in HSV-SAM by affinity chromatography. The effectiveness of the procedure depends in part upon obtaining antibody that reacts specifically with viral antigen and does not react with host-specific antigens. To achieve this, anti-CP-1 gamma globulin was thoroughly absorbed with uninfected BHK cell extracts (and fetal calf serum) until it reacted only against the CP-1 antigen.

Figure 4 illustrates the protein and radioactive profiles obtained when HSV-SAM labeled with tritiated glucosamine or valine was eluted from the immunosorbent column. HSV-SAM (30 mg of protein in 5 ml of 0.01 M phosphate buffer) was mixed for 2 hr at 25 C in a beaker with the CP-1 immunosorbent. The mixture was then poured into a column and washed with phosphate buffer until no more ultraviolet-absorbing material eluted (pooled fraction 1). The column was then eluted with 5 ml LiCl in 10% glycerol (the eluant was suggested by B. Del Villano, unpublished results) (pooled fraction 2). Fraction 2 contained approximately 1% of the total recovered protein. Each fraction was dialyzed at 4 C against 0.01 M phosphate buffer, pH 7.2, and concentrated. Gel diffusion (Fig. 5) illustrates that the immunosorbent selectively separated antigen CP-1 from the crude mixture of host and viral-induced antigens. Only one immunoprecipitin band was detected when fraction 2 was reacted against anti-SAM serum, and this band showed identity with fraction CP-1 antigen (isolated from Brushite) and a corresponding band in HSV-SAM.

Furthermore, only one band was observed when fraction 2 was reacted against absorbed anti-CP-1 serum or human gamma globulin. No host-specific immunoprecipitin bands were detected when unabsorbed anti-CP-1 serum was reacted against this fraction. Thus, as judged by immunological criteria, this technique apparently effected the isolation of the CP-1 immunoprecipitin from a mixture of viral-induced and host-specific antigens.

Table 1. HSV-neutralizing capacity of antiserum specific for various preparations

<table>
<thead>
<tr>
<th>Antiserum prepared against</th>
<th>Virus-neutralizing titer</th>
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</thead>
<tbody>
<tr>
<td>Virus-free soluble antigen mixture</td>
<td>1:840</td>
</tr>
<tr>
<td>CP-1 antigen</td>
<td>1:768</td>
</tr>
<tr>
<td>Human gamma globulin</td>
<td>1:512</td>
</tr>
</tbody>
</table>

Antisera were heated at 56 C for 30 min to inactivate complement.

Results are expressed as the greatest dilution of serum resulting in a 50% reduction of plaque-forming units as compared with appropriate virus-preimmune rabbit serum controls.
from the mg/ml. The effects of CP-1 on the serum by gamma globulin. We found also from HSV-SAM. The amounts of serum were 0.8 M, (0.1 M, pH 7.8) at a concentration of 100 µg of protein, completely blocked neutralizing activity. Far less CP-1 (10 µg of protein) isolated from the immunosorbent was required to block neutralizing activity of anti-SAM and anti-CP-1 sera. These results suggest that the fraction CP-1 (Brushite) and the antigen eluted from the immunosorbent (fraction 2) share the same biological activity as well as the same properties in gel diffusion.

**Evidence that CP-1 antigen is a glycoprotein.** Recent studies employing acrylamide gel electrophoresis have shown that several proteins from whole HSV-infected cells, as well as partially purified viruses and purified smooth membranes, are glycoproteins (11, 12, 19). Figure 6 shows the SDS-acrylamide gel radioactive pattern of the complete spectrum of 3H-glucosamine-labeled SAM proteins subjected to co-electrophoresis with 14C-valine-labeled SAM. The total spectrum of glycopeptides synthesized by HSV-HF-infected BHK cells form one major peak (peak 1, fraction 12 to 20) with two shoulders at fraction 20.

**FIG. 5. Gel diffusion analysis of fractions eluted from the immunosorbent prepared with anti-CP-1 gamma globulin.** Center well: anti-SAM serum. Peripheral wells: 1, fraction CP-1 (see Fig. 2) (protein concentration, 0.8 mg/ml); 2, uninfected BHK cell extract (protein concentration, 8.3 mg/ml); 3, pooled fraction 1 (see Fig. 4) (protein concentration, 5.0 mg/nl); 4, pooled fraction 2 (see Fig. 4) (protein concentration, 0.3 mg/ml).

Several additional features of the procedure are significant. (i) LiCl (5 M) did not elute all of the CP-1 antigen bound to the column. Additional amounts of CP-1 antigen were eluted with 10 ml of 0.05 M glycine-hydrochloride buffer, pH 3.2, or 0.05 M acetic acid. The eluates were diluted directly into a total of 20 ml of phosphate buffer (0.1 M, pH 7.8) to minimize any detrimental effects of acid on the antigen. (ii) The immunosorbent still retained its activity and suffered no apparent loss of specificity after being used several times. (iii) Small quantities of rabbit gamma globulin were also eluted from the immunosorbent. The latter component was detected by gel diffusion by employing goat anti-rabbit 7S serum. (iv) Since CP-1 immunoprecipitin was also found in fraction 1, we conclude that the capacity of the immunosorbent was not sufficient to effectively remove all of the CP-1 antigens from HSV-SAM.

**Blocking of the neutralizing capacity of antisera by various antigen preparations.** The following experiments were performed to determine the relationship between the neutralizing activity of anti-CP-1 serum and the immunoprecipitin band isolated from the immunosorbent. We reasoned that, if the CP-1 immunoprecipitin eluted with LiCl from the immunosorbent had the same biological activity as the fraction CP-1 isolated from calcium phosphate, then it should also be capable of blocking the neutralizing capacity of viral-specific antisera. We determined the capacity of various antigen preparations to block virus-neutralizing activity. Table 2 shows that extracts of uninfected BHK cells (800 µg of protein) had no effect on the neutralizing capacity of the sera tested. In contrast, HSV-SAM, at a concentration of 100 µg of protein, completely blocked neutralizing activity. Far less CP-1 (10 µg of protein) isolated from the immunosorbent was required to block neutralizing activity of anti-SAM and anti-CP-1 sera. These results suggest that the fraction CP-1 (Brushite) and the antigen eluted from the immunosorbent (fraction 2) share the same biological activity as well as the same properties in gel diffusion.

**TABLE 2. Blocking of serum-neutralizing capacity by different antigen preparations**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Protein (µg)</th>
<th>Anti-SAM serum (%) blocking</th>
<th>Anti-CP-1 serum (%) blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected BHK cell extract</td>
<td>800</td>
<td>0b</td>
<td>0</td>
</tr>
<tr>
<td>HSV-soluble antigen mixture</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>CP-1 antigen&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>80</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Anti-CP-1 serum prepared to calcium phosphate fraction 1 (see Fig. 2).

<sup>b</sup> Not blocking serum-neutralizing capacity.

<sup>c</sup> Isolated from an anti-CP-1 immunosorbent (see Fig. 4).
17 and 19 and three smaller peaks of radioactivity (peak 2, fractions 21 to 25; peak 3, fractions 25 to 28; peak 4, fractions 29 to 34). The estimated molecular weights (21, 34) for these glycopeptides are peak 1, 100,000; peak 2, 76,000; peak 3, 70,000; and peak 4, 50,000. Although recent reports indicate that certain polypeptide chains covalently linked to carbohydrate migrate at rates which are not proportional to their molecular mass (2, 20), this does not appear to be true for herpesvirus glycoproteins (23). The general profile of 3H-glucosamine-labeled proteins obtained here strongly resembles the pattern of glycoproteins reported by Olshesky and Becker (11), as well as the pattern seen in Hep-2 cells infected with HSV strain F (8).

To determine whether the CP-1 antigen was a glycoprotein, BHK cells were labeled with 3H-glucosamine 3 hr after infection with HSV and harvested 14 hr later. Newly synthesized HSV-specific proteins are preferentially glycosylated during this interval (11, 22). HSV-SAM was prepared as described in Materials and Methods and passed through the CP-1 immunosorbent (see Fig. 4) to separate the CP-1 immunoprecipitin from the remaining viral and host antigens. Tritiated glucosamine-labeled CP-1 antigen, isolated from the immunosorbent, was denatured by treatment with SDS, urea, and mercaptoethanol and subjected to SDS-acrylamide electrophoresis (Fig. 7A). The radioactive profile indicates the presence of a number of glycopeptide bands. However, for the purpose of this report, we will consider only three areas: peak 1 (fraction 13 to 20, estimated molecular weight of 100,000), peak 2 (fraction 30 to 35, estimated molecular weight of 50,000), and the plateau of radioactivity observed between peak 1 and 2 (estimated molecular weight of 70,000). Fig. 7B shows the radioactive profile obtained when 3H-valine-labeled CP-1 antigen (Fig. 4) was treated in the same manner as 3H-glucosamine-labeled antigen. A comparison of the two patterns (Fig. 7A and B) indicates that the CP-1 antigen is composed of a number of polypeptides migrating to the same relative positions as the glycopeptides. Since the radioactive profiles are similar, we suggest that the CP-1 antigen (LiCl fraction), which displays a less complex radioactive profile than that shown by HSV-SAM (Fig. 6), is associated with carbohydrate. However, further studies are necessary to adequately characterize the CP-1 antigen.

Evidence which suggests that the 3H-glucosamine was incorporated directly into the CP-1 immunoprecipitin is as follows. Tritiated glucosamine-labeled CP-1 (from the immunosorbent) was reacted in gel diffusion with anti-CP-1 serum. After development of the pattern, the agar was washed repeatedly with saline, and the immunoprecipitin band was carefully excised and counted in Aquasol. Gel slices containing the specific immunoprecipitin band possessed 98 counts per min of tritium label (an average of four different im-
munoprecipitin bands) above control gel sections (average of 25 counts per min per slice) cut from areas adjacent to the immunoprecipitin band on the same slides. The following experiment was performed to show that the radioactive material found in the band was due to the viral-specific, antigen-antibody reaction and not to radioactive material trapped artificially by the precipitin band. Tritiated glucosamine-labeled CP-1 was added to a gel diffusion well containing preimmune rabbit serum. The mixture was then reacted against goat anti-rabbit gamma globulin. Background values of radioactivity (25 counts per min per immunoprecipitin band) were obtained in the immunoprecipitin band indicating no trapping of labeled material. These studies reinforce the evidence that there is carbohydrate associated with the viral-specific CP-1 antigen.

**DISCUSSION**

Our study provides a relatively simple method for the isolation of a virus-specific antigenic moiety (CP-1) from a mixture of viral-induced antigens and host cell antigens. The separation occurs because the CP-1 antigen absorbs poorly to a Brushite column and elutes at lower phosphate concentrations than the remaining viral-induced antigens and most of the host proteins.

Calcium phosphate chromatography represents a good method for the separation of HSV proteins. However, although this method separated the CP-1 immunoprotein from detectable quantities of other antigens, it did not result in complete purification of CP-1 according to physical-chemical criteria. We were, however, able to utilize CP-1 to prepare a specific antiserum. Since we could not demonstrate conclusively that anti-CP-1 serum contained no antibodies to antigens other than CP-1, we subscribe to the convention set up by Watson and Wildy (32) to refer to the antiserum as monoprecipitin rather than monospecific.

The need for monoprecipitin sera has been emphasized often (12, 30, 32, 33). As pointed out by Watson and Wildy (32), for example, studies of the cellular location of HSV-specific antigens by fluorescence microscopy, as well as kinetic studies on the appearance of the antigens during the growth cycle of the virus, are hampered by the polyvalency of the antisera.

Several lines of evidence suggest that the CP-1 immunoprecipitin is a viral-specific antigen. First, specific antiserum which had been repeatedly absorbed with uninfected BHK extracts and serum still retained the capacity to react in gel diffusion with CP-1 antigen. Secondly, anti-CP-1 serum reacted in gel diffusion with HSV-SAM to yield one precipitin band in identity with the band formed against human gamma globulin, as well as the band formed against CP-1. If the CP-1 fraction had contained infectious virus, noninfectious virus, or more than one soluble antigen, one would have expected to find antibodies produced to these viral products. If there were other viral-specific antigens present in CP-1, we were unable to detect them by our methods. Finally, the CP-1 immunoprecipitin stimulated the production of HSV-neutralizing antibody of high capacity. This last observation suggests that CP-1 is a virus structural component which is associated with the envelope of the virus. Alternatively, a host protein associated with the viral envelope rather than the viral-specific CP-1 antigen could be involved in virus neutralization. Evidence to support this alternative was presented by Watson and Wildy (31), who showed that anti-host cell serum agglutinated the envelope particles of HSV. However, no neutralization data were presented by these authors. Becker and Olshovsky (11) also found cellular proteins in preparations of the enveloped virus. On the other hand, Spear and Roizman (23) recently presented evidence that cellular proteins are probably not components of highly purified mature virions. We believe it is unlikely that the neutralizing activity associated with CP-1 antigen was due to a host-derived protein since antisera to this antigen did not react with normal BHK components by immunodiffusion. In addition, large amounts of uninfected BHK protein failed to block the neutralizing capacity of anti-CP-1 or anti-SAM serum, whereas very small amounts of CP-1 blocked these sera completely. It was unlikely that the serum-blocking effect of the CP-1 preparation was due to virus, since no infectious virus could be detected in the plaque assay nor were any virus particles observed by electron microscope examination of CP-1 (unpublished results).

The hyperimmune antisera used in these experiments are probably comparable to "late" 7S antibody described by Hampare (7). Late 7S antibody has specificity for virion antigens associated with the virus envelope surface (as well as for other antigens), and these antigens are readily accessible for neutralization in the absence of complement. Considering the structural complexity of the virus envelope, it is possible that more than one virus-specific antigen is involved in the neutralization of the virus. In this regard, Hampare et al. (7) observed that HSV neutralization can also occur with antibodies reactive with subsurface antigens. Rowlands et al. (18) showed that two distinct immunogenic sites on the surface of foot-and-mouth disease virus are concerned with the production of two distinct neutralizing antibodies. Although we found a definite relation-

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ship between the virus-neutralizing activity of the anti-CP-1 serum and the CP-1 immunoprecipitin, further experimentation will be necessary to determine if this is the only HSV antigen responsible for this biological activity.

We have presented evidence which suggests that the CP-1 immunoprecipitin is, in part, glycoprotein. Glycoproteins found on the cellular membranes after infection with HSV have been associated with a variety of biological phenomena. These include alteration of the social behavior of cells (12), hemadsorption (29), and immunologic injury or cytolytic effect (13, 16). Roizman (12) has reviewed the evidence indicating that the "new" antigen(s) found on the surface of HSV-infected cells is a structural component of the virus envelope. Furthermore, all of the glycoproteins found in the mature HSV apparently are associated with the envelope of the virus (11, 23). It was also observed that there is a strong correlation between neutralizing antibody titer and cytolytic (15) and hemadsorption-inhibition titers (29), suggesting that the antigen which stimulates production of neutralizing antibody may also be responsible for these other phenomena. Since fraction CP-1 stimulated neutralizing antibody, it may be hypothesized that the antigen CP-1 is responsible for some or all of the altered properties of the infected cells. The monoprecipitin-CP-1 serum should be useful for studying these phenomena.

On the basis of SDS-polyacrylamide gel electrophoresis, the CP-1 antigenic fraction isolated from the immunosorbent appeared to contain a number of radioactively labeled polypeptides and glycopeptides. This observation may be explained in several ways. (i) The CP-1 antigen derived from HSV-SAM was a complex or aggregate of several viral-specific proteins. When the radioactive HSV-SAM was passed through the immunosorbent, the CP-1 antigen combined with the specific anti-CP-1 antibody and carried the contaminating or complexed proteins with it. (ii) The anti-CP-1 serum used for preparation of the immunosorbent was polyvalent and contained low concentrations of antibody to several virus structural components other than CP-1. Low concentrations of several radioactively labeled, viral-specific proteins would attach to the immunosorbent and after elution would be detected by acrylamide gel electrophoresis, but not by immunodiffusion. (iii) The CP-1 antigen was made up of a number of different subunits or molecular forms containing varying amounts of lipid or carbohydrate residues.

Further studies are now in progress to purify and characterize the CP-1 immunoprecipitin.

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LITERATURE CITED