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Isolation and Characterization of Monoclonal Antibodies to Structural and Nonstructural Herpesvirus Saimiri Proteins

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An improved screening procedure was applied to identify hybridomas secreting antibodies to herpesvirus saimiri-specified polypeptides among the products of fusions between SP2/0 myeloma cells and spleen cells from mice immunized with purified virus particles or virus-specific DNA-binding proteins. Twenty-four monoclonal antibodies were isolated with specificities for 13 different virus-specified polypeptides (or complexes of polypeptides), including the major capsid protein of the virus (150K), the 160K and 130K structural proteins, a 108K structural phosphoprotein, structural glycoproteins, the nonstructural early 76K protein, early nonstructural DNA-binding proteins of 48 to 51K and 110K and the major immediate-early protein of 52K. Antibody to the virus 76K protein precipitated a host protein of 62K, and a number of antibodies specific for host proteins were also isolated. Antibody to the 52K immediate-early polypeptide precipitated the delayed-early 76K protein, whereas the antibody to the 76K protein did not precipitate the 52K polypeptide. These observations suggest the presence of epitopes common to virus and host proteins and an antigenic site common to an immediate-early and a delayed-early virus protein. The antibodies were used to examine the sites of intracellular accumulation of virus polypeptides, the formation of complexes of structural proteins, and the postsynthetic processing of virus proteins. The present collection of monoclonal antibodies provides a set of reagents with specificities for members of each of the major kinetically or functionally distinct classes of virus gene products.

The synthesis of a novel virus thymidine kinase activity, a phosphonoacetic acid (PAA)-sensitive DNA polymerase activity, and more than 30 virus-specified polypeptides can be detected in infected cells during a productive cycle of herpesvirus saimiri (HVS) replication (8, 16, 18, 23). We have begun a detailed characterization of the regulation and properties of these virus gene products. In the presence of concentrations of PAA that inhibit the synthesis of virus DNA, a distinct subset of early virus-specified polypeptides whose synthesis was insensitive to PAA (e.g., 110K, 76K, 48 to 51K, 29 to 31K, 24K, and 21K) could be differentiated from late proteins (19), the synthesis of which was sensitive to PAA (e.g., 220K, 160K, 150K, 130K, 117K, 38K, 32K, and 12K). The early group of virus proteins includes nonstructural DNA-binding proteins of 110K and 48 to 51K (2) and a major immediate-early protein of 52K (23a). The late group includes the major polypeptides of the virus capsid (e.g., 150K), nonglycosylated noncapsid components of the virion (160K and 130K), as well as the multiple glycosylated species in the infected cell and the virus particle (i.e., 170 to 220K, 125 to 145K, 117K, 83 to 88K, 65 to 75K, 52 to 58K, 25 to 27K, and 12.5 to 13K) (unpublished observations; 10, 21, 22).

The power of specific antisera as tools to associate functions with protein structure is well established. In our previous studies we have prepared monospecific rabbit antisera to the virus-specific polypeptides of 160K and 28K that are selectively released into the culture medium from infected cells. The anti-160K antibody neutralized virus infectivity (22), and the anti-28K antibody was used to show the presence of a nuclear 30K precursor to a cytoplasmic 28K product (23). However, polyclonal reagents are not ideal for structure-function studies; the biologically relevant effects of an apparently monospecific serum may be mediated by an undetected contaminating minor antibody population with an independent specificity. To provide reagents for the more sensitive and specific detection and characterization of HVS polypeptides we have therefore isolated a collection of monoclonal antibodies against structural and nonstructural proteins of HVS. In this paper we present an improved rapid procedure for the screening of antibodies secreted by hybridoma cell lines and the characterization of more than 20 monoclonal antibodies, including specificities for at least 13 HVS-specified polypeptides, that we have isolated by this procedure.

MATERIALS AND METHODS

Cells and viruses. A continuous line of owl monkey kidney cells (OMK-210; 23) was used throughout for virus growth and infectivity titrations. Cells were grown at 37°C as monolayers in 25- or 75-cm^2 disposable tissue culture flasks or in rotating 80-oz. (ca. 2,500-ml) Winchester bottles with Dulbecco H21 modification of Eagles tissue culture medium supplemented with 10% newborn calf serum. The attenuated derivative of HVS strain 11 (HVS-11Att; 28) was used for most of the experiments reported in this paper. In addition, HVS strain KM744 (23) was used in some comparative studies. For the preparation of homogenates of infected and mock-infected cells for use as antigens (below), monolayers of OMK cells were infected with 5 PFU of HVS-11Att per cell or were mock infected and incubated at 37°C for 48h (23). Infected and mock-infected cultures were then washed twice, harvested into ice-cold phosphate-buffered saline, disrupted by ultrasonic oscillation, and stored at −70°C unless used immediately.

Immunization of mice, fusion of splenocytes with SP2/0 cells, selection of antibody-secreting hybridomas, and nomenclature for monoclonal antibodies. Inbred BALB/c mice were

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immunized with purified virions (21) of HVS-11Att (250 μg of protein per immunization) or with an alum precipitate (15) of proteins from the fractions eluted by 0.4 M NaCl during DNA-cellulose affinity chromatography of polypeptides from virus-infected cells (30 μg of protein per immunization). An analysis of the polypeptides of this DNA-binding protein fraction is shown in Fig. 2, and a detailed account of the methods used has been given previously (2). At the same time mice were immunized with a killed and fixed suspension of *Bordetella pertussis* (2×10^9 cells per mouse) to enhance the immune response (12). After 28 days, mice were reimmunized intraperitoneally, and after a further 21 days they were boosted by both an intraperitoneal and a tail vein immunization. After 3 days spleens were removed, the splenocytes were fused with SP2/0-Ag14 myeloma cells (29), and the hybrid cells were plated in 96-well microtiter plates so that approximately one colony of cells grew in every other well under selective growth conditions. Hybrid cell culture conditions were based on those previously described (4, 13).

A novel screening procedure was used to identify hybridomas secreting specific antibodies to virus or host antigens. In this procedure, sheets of nitrocellulose (BA 85, 0.45 μm; Schleicher & Schull, West Germany) were cut to the size of 96-well microtiter plates and incubated with infected or uninfected total cell antigen. Each sheet was agitated for 15 min at 20°C with homogenates from 4×10^7 infected or uninfected cells diluted to 20 ml with phosphate-buffered saline. The nitrocellulose sheets were then washed three times with 3% bovine serum albumin in Tris-buffered saline (50 mM Tris-hydrochloride [pH 7.4], 0.1 M NaCl) or with 0.5% Tween 20 in Tris-buffered saline. The bovine serum albumin or nonionic detergent was included in the buffer to saturate all of the protein binding sites on the sheets of nitrocellulose (1). Samples (150 to 200 μl) of the culture medium from each hybridoma clone were transferred to 96-well microtiter plates, and a sheet of nitrocellulose with infected or uninfected cell antigens was placed over the plate. An empty microtiter plate was inverted over the plate with the test samples, and the two plates were clamped tightly together, making a sandwich of the antigen bound to nitrocellulose paper. This assembly was inverted, and the samples of culture medium were shaken over the nitrocellulose sheet for 2 h at 37°C. The sheets were then removed and washed three times in Tween buffer (0.2% Tween 20, 50 mM Tris-hydrochloride [pH 7.4], 0.1 M NaCl), and the bound antibodies were detected by sequential reactions with ^{125}I-labeled protein A and then by an enzyme-linked immunosorbent assay with an antibody to total mouse immunoglobulins. The nitrocellulose sheets were incubated at 37°C for 30 min with ^{125}I-labeled protein A (5×10^6 cpm in 10 ml of Tween buffer) and then washed three times in Tween buffer. These same sheets of nitrocellulose were then incubated for 1 h at 37°C with peroxidase-conjugated rabbit anti-mouse immunoglobulin antibody (Nordic, Maidenhead, United Kingdom) diluted 1/200 in Tween buffer and washed twice with Tween buffer and twice with distilled water. The nitrocellulose was immersed in a dianisidine substrate solution for 10 min at room temperature, and the presence of peroxidase conjugated antibody was indicated by the formation of an insoluble brown precipitate as the product. The substrate was made by dissolving 0.1 g of θ-dianisidine (Sigma, Dorset, United Kingdom) in 0.5 ml of absolute ethanol, diluting in 20 ml of distilled water, filtering, and adding hydrogen peroxide to 0.06%. Nitrocellulose sheets were then washed twice more with distilled water, dried, and exposed to X-ray film to detect bound ^{125}I-labeled protein A.

Hybridomas secreting specific antibody to virus or cell antigens were subcloned by plating cells from the original hybridoma cell line in 96-well microtiter plates to give approximately 1 cell per well in one microtiter plate and 1 cell in every 5th, 25th, or 125th well in subsequent microtiter plates. These plates were examined with an inverted microscope after 12 h, and wells containing single cells were marked. Single hybridoma colonies were taken after a further 10 to 14 days from those wells containing single cells at 12 h and from plates that had fewer than 25 colonies per 96-well plate. Colonies that continued to secrete specific antibody were used to produce ascitic fluids by intraperitoneal inoculation of 10^6 to 10^7 cells in BALB/c mice that had been pretreated with 0.5 ml of 2, 10, 14-tetramethylpentadecane 6 to 10 days previously. Each monoclonal antibody was given a trivial two-letter name. The first letter was S or D to denote hybridomas originating from mice immunized with purified virions (S) or purified preparations of virus DNA-binding proteins (D). The second letters were identifying labels for individual antibodies. These trivial names were subsequently amplified by the addition of information on the specificity of the antibody when this became known; e.g., antibody DC recognizes the 110K DNA-binding protein, and its full name is DC(110K).

Preparation of radiolabeled antigen extracts, immune precipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cell monolayers in 75-cm^2 tissue culture flasks or in rotating 80-oz. Winchester bottles were infected with 1 to 10 PFU of HVS per cell. After an adsorption period of 2 h at 37°C, the inoculum was removed and replaced with tissue culture medium containing 10% newborn calf serum. Infected or mock-infected cultures were labeled with either L-[^{35}S]methionine (500 Ci/mMol; Amersham International) in tissue culture medium (5 μCi/ml) with 0.1 of the normal concentration of unlabeled methionine or with $^{32}P_i$ (carrier free; Amersham International) in phosphate-free tissue culture medium (5 μCi/ml). At the end of the labeling intervals, cultures were removed, washed with ice-cold phosphate-buffered saline, and pelleted. Cell pellets were suspended in immune-precipitation buffer (20 mM Tris-hydrochloride [pH 7.2], 5 mM EDTA, 1% Nonidet P-40, and 0.65 M NaCl; 4×10^6 to 10×10^6 cells per ml of buffer) by sonication with an ultrasonic probe. Soluble antigen extracts were obtained after pelleting the particulate material from these total cell antigen extracts by centrifugation (13,500 × *g* for 30 min). Immune complexes were formed by incubating 0.2-ml samples of the soluble antigen fractions with an excess of antibody (1 μl of undiluted ascitic fluids) for 2 h at 4°C. Immune complexes were isolated on an excess of a fixed suspension (11) of the Cowan A strain of *Staphylococcus aureus* (20 μl of a 10% [wt/vol] suspension per μl of ascitic fluid for 30 min at 4°C), washed three times by suspension and sedimentation (3,300 × *g* for 3 min) from immune precipitation buffer containing 10% sucrose, removed from the bacterial immunoabsorbent by heating (80°C for 5 min) in gel electrophoresis sample buffer (0.05 M Tris-hydrochloride [pH 7.0], 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 5% glycerol), and the dissociated polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23). After electrophoresis the polyacrylamide gels were stained with Coomassie brilliant blue (total protein stain), destained in acetic acid-methanol, and dried. Labeled polypeptides were visualized by autoradiography with Fuji X-ray film.

Western blots. Polypeptides separated on polyacrylamide gels were transferred to sheets of 0.45-μm nitrocellulose by

transverse electrophoresis (26, 27). After transfer the filters, bearing samples of the electrophoretically separated polypeptides, were washed for 12 h at 20°C with 4 M urea in phosphate-buffered saline containing 0.2% Tween 20 and then for at least 4 h with 0.2% Tween in phosphate-buffered saline (1). The filters were then incubated with antibody (10 μl of ascitic fluid in 1 ml of immune precipitation buffer) for 1 h at room temperature and then washed three times with immune precipitation buffer. Antibody bound to nitrocellulose sheets was detected with ^{125}I-labeled protein A as described above.

Immunofluorescence and purification of immunoglobulins. OMK cells, grown on cover slips or glass slides, were mock infected or infected with 2 to 10 PFU of HVS-11Att per cell. At 24 h after infection, cells were fixed with formaldehyde and penetrated with Triton X-100 (17). Purified preparations of monoclonal antibodies (see below) were used to stain cells by indirect immunofluorescence with rhodamine-conjugated goat anti-mouse immunoglobulin as the second antibody. Cells were simultaneously stained with 2 mM 4′-6-diamidino-2-phenylindole (DAPI; 25), which binds to DNA and thus stains cell nuclei. Stained cells were observed with a Zeiss photomicroscope (×25 or ×40 objective; UV excitation and blue fluorescence for DAPI, green excitation and red fluorescence for rhodamine), and photographs were taken on Kodak Ektachrome 400 film with exposure times of 30 to 120 s for rhodamine fluorescence and 1 to 2 s for DAPI fluorescence.

Monoclonal antibodies were purified from ascitic fluids by absorption onto immunoabsorbent monolayers of *S. aureus* Cowan strain A formed on 25-cm^2 tissue culture flasks (20). Excess antibody (50 μl of ascitic fluid in 1 ml of phosphate-buffered saline) was irrigated over the immunoabsorbent monolayers for 20 min at 20°C, and unbound proteins were removed by washing the monolayers twice with distilled water. Bound antibody was eluted from the monolayers by 0.1 M citrate buffer (pH 3.5; 1 ml per 25-cm^2 monolayer), and the solutions, containing 10 to 20 μg of purified antibody, were titrated to neutral pH with 3 M Tris-hydrochloride (pH 8.5) before being used in immunofluorescence tests.

RESULTS

Preliminary screening of hybridoma culture for antibodies to virus-specific proteins. A number of techniques have been widely used for the screening of hybridoma cell lines for the production of antibodies to virus-specified proteins (e.g., immune precipitation and gel electrophoresis, immunofluorescence, virus neutralization, immune electroblotting, and enzyme-linked immunosorbent assay or radioimmunoassay on virus antigens in fixed monolayers of infected cells). However, these methods are either lengthy and cumbersome (e.g., immune precipitation and gel electrophoresis) or highly selective in terms of the classes of antibodies they detect (e.g., virus neutralization, immune electroblotting). We have developed a simple, sensitive, rapid and general screening procedure that we have applied throughout the present work. In this method, duplicate sheets of nitrocellulose were saturated with homogenates of total infected or mock-infected cells, mounted between 96-well microtiter plates, and reacted with 96 0.15-ml samples of culture media from hybridoma cultures. Figure 1 illustrates results from a typical set of primary tests on cultures from fusions of SP2/0 myeloma cells with spleen cells from mice immunized with purified virus particles (Fig. 1a) or immunized with purified DNA-binding proteins (Fig. 1b) and secondary tests on

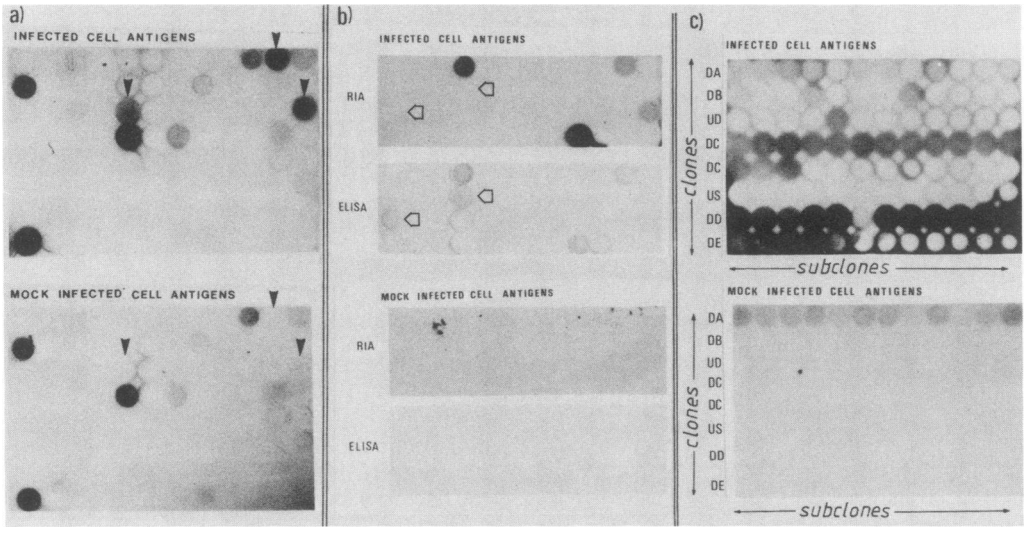

FIG. 1. Illustration of the method used to screen culture media from hybridomas for antibody to virus or host-specific-antigens. (a) Culture media from hybridomas isolated after fusions with splenocytes from mice immunized with purified virus, tested against antigens of infected and mock-infected cells. Bound antibody was detected by radioimmunoassay with ^{125}I-labeled protein A; clones secreting antibodies directed against virus-specific antigens are readily identified (filled arrows). (b) Sequential detection both of protein A binding antibodies (radioimmunoassay) and of total antibodies (enzyme-linked immunosorbent assay with anti-mouse immunoglobulin) among antibodies bound to single sheets of nitrocellulose bearing antigens from either infected or mock-infected cells. The culture fluids were from hybridomas arising after fusions with spleen cells from mice immunized with purified virus-specific DNA-binding proteins, and all clones detected in this example are secreting antibodies to virus-specific antigens. The open arrows indicate those clones secreting antibodies that do not bind protein A. (c) Application of the radioimmunoassay with ^{125}I-labeled protein A for the detection of antibodies in the culture media from subclones of hybridomas selected from the initial screening procedure as reactive with host (DA) or virus (DB, DE) antigens. UD and US indicate subclones all of which are negative (US) or which were lost on further passage (UD).

subclones of a number of cell lines selected for further analysis (Fig. 1c). Clearly, the test permits rapid and unequivocal differential identification of clones secreting antibodies to components of the uninfected cell (Fig. 1, spots common to wells in Fig. 1a; clone DA and its subclones in Fig. 1c) from those secreting antibodies specific for components of infected cells and absent from uninfected cells (Fig. 1a, arrows). Antibodies bound to sheets of nitrocellulose were detected by sequential reactions with ^{125}I-labeled protein A and then with an enzyme-linked immunosorbent assay test with an antibody to total mouse immunoglobulins, thus permitting immediate detection and discrimination of protein A-binding and nonbinding classes of mouse immunoglobulins (Fig. 1b).

By applying these tests, 70 of 600 hybridomas established after fusions with splenocytes from mice immunized with purified virus DNA-binding proteins were identified as secreting antibody to virus proteins; 22 of these antibodies bound to protein A. Of these same 600 hybridomas, only 6 were shown to secrete antibody reactive with antigens of uninfected cells. In contrast, 350 hybridomas established after fusions with splenocytes from mice immunized with purified virus particles, 51 were found to secrete protein A-binding antibodies and of these only 15 were directed against virus-specific antigens, whereas 36 reacted with antigens of uninfected cells. Because the ability of antibody to bind protein A is such a useful property, the majority of hybridomas that were subcloned and characterized were those that secreted specific antibody that also bound to protein A. Of 40 hybridomas selected for further analysis, subclones of 13 either did not grow or failed to secrete specific antibody after subcloning.

Characterization of antibody specificities: immune precipitation reactions with extracts of infected and mock-infected cells. Samples of all monoclonal antibodies were used in immune precipitation reactions against extracts from HVS-infected and mock-infected cells labeled with [^{35}S]methionine, and the precipitated polypeptides were analyzed by gel electrophoresis and autoradiography. Representative sets of results are shown in Fig. 2, 3, and 4. The specificities of many of the monoclonal antibodies were readily identifiable from these results. For example, SA clearly reacts with an 11K virus protein (Fig. 2 and 3), SC reacts with a 108K protein, SH and SJ react with the 160K protein, DC, DD, DF, DJ, DL, DM, DP, and DS react with the 110K DNA-binding protein, and DI reacts with the 48 to 51K DNA-binding protein (Fig. 2b). In cases where independent antibodies precipitated the same polypeptide (e.g., those reacting with 110K, above), evidence that they represent products of clonally unrelated hybridomas was provided by the characteristic differences in the electrophoretic mobilities of their heavy and light chains (Fig. 2a; compare also SD with SE and SF with SG).

In a number of cases a single monoclonal antibody precipitated multiple virus-specific polypeptides (Fig. 2; antibodies SB, SD and SE, SF and SG, DB, DH), virus and host polypeptides (Fig. 2; DE, DO, SI), or multiple host polypeptides (Fig. 4; DK and DN). These may represent (i) instances where cloning failed to separate hybridomas secreting antibodies with independent specificities, (ii) the existence of complexes between independent virus or virus host polypeptides such that antibody to one component also coprecipitates antigenically unrelated members of the complex, (iii) the existence of shared epitopes on noncomplexed proteins which are related as precursor and product and which therefore share extensive sequence relatedness, (iv) the existence of shared epitopes due to limited regions of homologous structure in otherwise unrelated proteins, and (v) the existence of specific nonimmune binding interactions between antibodies and virus or host proteins. The initial cloning procedure makes the persistence of mixed clones highly improbable, and the characteristic electrophoretic mobility of the heavy and light chains of the secreted immunoglobulins also constitutes a criterion of monclonality (Fig. 2a). Moreover, in all instances where we have observed multiple species in immune precipitation reactions, the same patterns of reactivity have been produced after further subcloning of the hybridomas concerned (Fig. 5). It therefore seems that the presence of multiple antibody specificities is not the explanation for our results.

The polypeptides precipitated by antibody DB (Fig. 2b) correspond in both number and relative molarity to the components of a capsid structure that we have isolated from similar extracts of HVS-infected cells, and we have independent evidence that the components of this complex have distinctive properties and are unlikely to share antigenic determinants (2; unpublished observations). We therefore believe that this antibody recognizes an epitope on this capsid structure. In the cases of the pairs SD and SE and SF and SG, independent monoclonal antibodies precipitate the same polypeptides, which share some of their known properties. Thus the electrophoretically heterogeneous 45 to 52K and 65 to 75K polypeptides precipitated by SD and SE and the 125 to 145K, 117K, 82 to 92K, and 69 to 76K polypeptides precipitated by SF and SG are all known to be glycosylated (22; this paper and unpublished results). We therefore believe that at least some of the proteins precipitated by these antibodies are likely to share sequences and multiple antigenic sites or to be part of a stable complex of functionally related proteins. Antibody SD reacts with both 45 to 52K and 65 to 75K polypeptides on a Western blot (see below), and in this case it is clear that the epitope is present on both of these polypeptides.

A number of other antibodies precipitate multiple virus proteins with different properties or virus and host proteins. The most interesting example is provided by antibody SB, which precipitated major virus-specific polypeptides of 76K, 50 to 52K, and 31.5K and minor polypeptides of 86K and 40 to 41K from extracts of cells labeled throughout a productive cycle of virus growth (Fig. 2b). We have previously shown that the 52K polypeptide is the major immediate-early protein of HVS and is precipitated by this antibody in the absence of the 76K protein, which is a delayed-early virus gene product (23a). An independent monoclonal antibody to the 76K polypeptide, SI (Fig. 2b), precipitates the 86K and 31.5K polypeptides, but does not precipitate the 50 to 52K or 40 to 41K polypeptides. Moreover, the 76K protein is distributed between nuclear and cytoplasmic fractions of infected cells, whereas the 50 to 52K protein accumulates in the nuclear fraction. Immune precipitates formed with the SB monoclonal antibody and cytoplasmic fractions of infected cells show a marked enrichment in the precipitation of the 76K polypeptide relative to the 50 to 52K polypeptides (data not shown). In addition, the 76K polypeptide is not a phosphoprotein, whereas the 50 to 52K polypeptide is (see Fig. 7). Secondary subcloning of the SB hybridoma produced multiple clones with specificities indistinguishable from the parent cell line (Fig. 5). The present evidence therefore suggests that these coprecipitated polypeptides are not complexed, but share some antigenic sites in common.

The precipitation of the host 62K polypeptide by antibody SI likewise persists on recloning (Fig. 5), but is not invari-

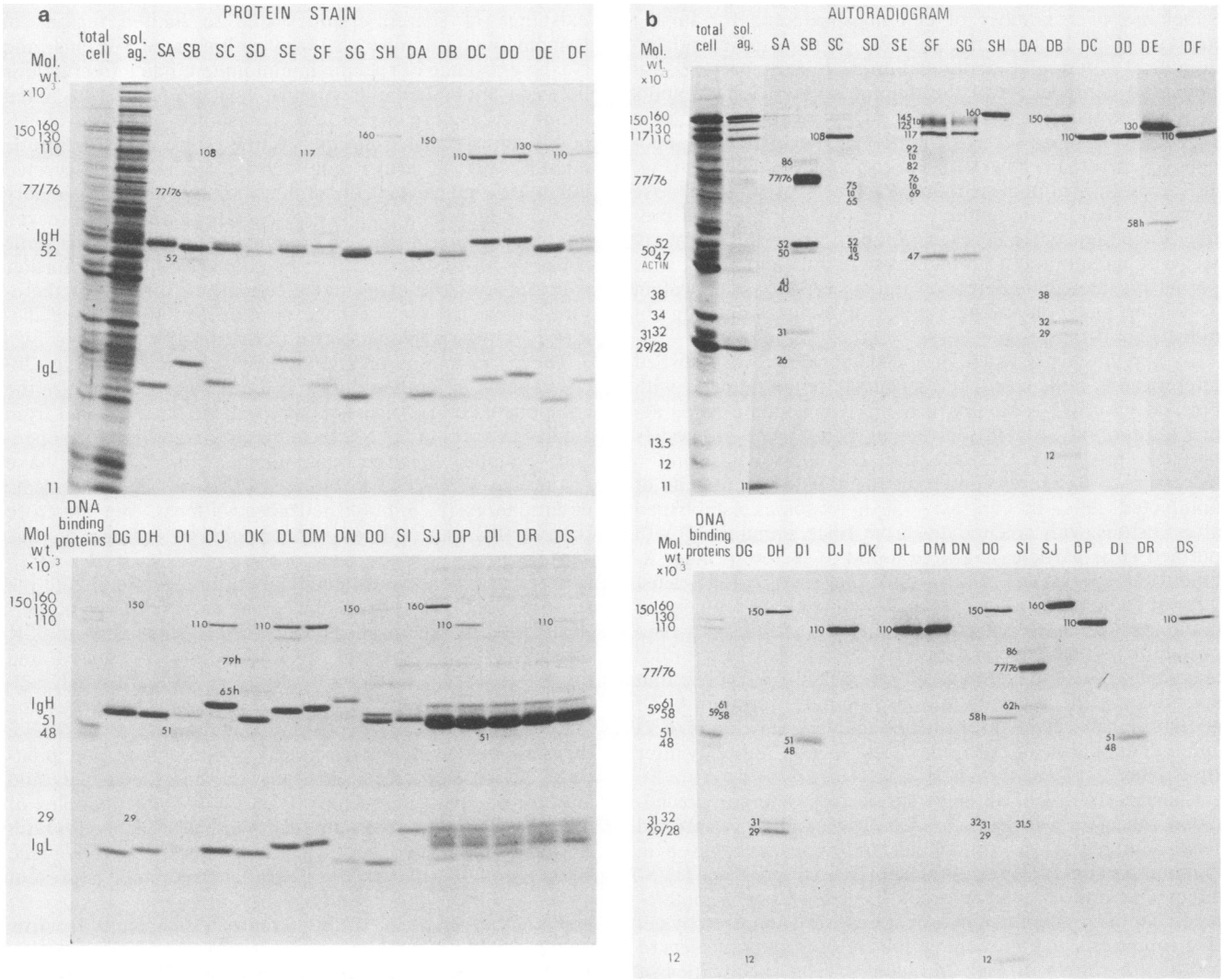

FIG. 2. Analysis of (a) total polypeptides (Coomassie brilliant blue-stained gel film) and (b) [^{35}S]methionine-labeled polypeptides (autoradiogram) separated by electrophoresis through a 13% polyacrylamide slab gel from immune precipitates formed by the reaction of monoclonal antibodies (SA to DS) with a soluble antigen extract from infected cells (upper panel, track 2). Also shown are polypeptides from samples of a total infected cell lysate (upper panels, track 1) and from a purified preparation of virus DNA-binding proteins (lower panels, track 1). Monoclonal antibodies SA to SI bound protein A, and thus the immune complexes were isolated on *S. aureus* (see the text). Monoclonal antibodies SJ to DS did not bind, or bound poorly (DI) to protein A, and the immune complexes were isolated on *S. aureus* saturated with sheep anti-mouse Fab_2 antibody. The heavy (immunoglobulin H [IgH] and light (IgL) chains of sheep immunoglobulins in these tracks therefore obscure the heavy chains and some of the light chains arising from the monoclonal antibodies. In the remaining samples (SA to SI) characteristic differences in the electrophoretic mobilities of heavy and light chains can be detected between most of the monoclonal antibodies (protein stain). Virus- and host-specific polypeptides represented in immune precipitates are annotated with their apparent molecular weights ($\times 10^3$), host proteins are indicated by the suffix h (e.g., 79h with antibody DK in the protein stained gel, 62h with antibody SI in the autoradiogram).

ably associated with the 76K polypeptide and is precipitated from uninfected cell lysates in the absence of the 76K polypeptide. Immune precipitations performed under mildly denaturing conditions (0,1% SDS, 1% sodium deoxycholate, 1% Nonidet P-40) show that reactivity with the host 62K protein is more resistant than is reactivity with the virus 76K protein (results not shown). The precipitation of the host 62K polypeptide is a property we have only observed with this single monoclonal antibody. In contrast, a number of antibodies of independent and unrelated specificities precipitate a 58K host protein from both infected and uninfected cells (e.g., DE, DO, and DN), and we have found that this protein is a common nonspecific component of many immune precipitates formed under less stringent conditions (i.e., 0.15 M NaCl). In addition, monoclonal antibodies that precipitate the 58K host polypeptide in immune precipitation reactions did not react with uninfected cell antigens bound to sheets of nicrocellulose paper (e.g., DE; Fig. 1c). We therefore believe that the evidence suggests a shared epitope reacting with the SI antibody on the host 62K and virus 76K polypeptide, but that the precipitation of the 58K host protein may be the result of a nonimmune interaction

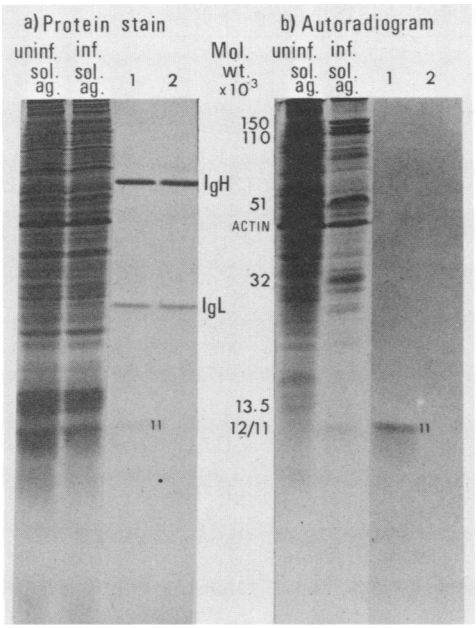

FIG. 3. Analysis of (a) total polypeptide (Coomassie brilliant blue-stained gel film) and (b) [^{35}S]methionine-labeled polypeptides (autoradiogram) separated by electrophoresis through a 16% polyacrylamide slab gel from immune precipitates formed by the reaction of the SA monoclonal antibody with soluble extracts of infected (track 1) or uninfected (track 2) cells.

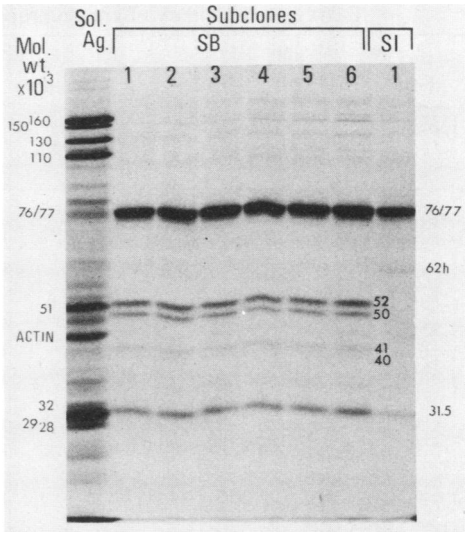

FIG. 5. Analysis of [^{35}S]methionine-labeled polypeptides, separated by electrophoresis through a 10% polyacrylamide slab gel, from immune precipitates formed by the reaction of antibody from six independent secondary subclones of hybridoma SB and one secondary subclone of hybridoma SI with a soluble antigen extract (track 1) from infected cells. Polypeptides are annotated with their apparent molecular weights ($\times 10^3$). The subclones of SB each precipitate multiple virus-specific polypeptides (31.5 to 76K), and the subclone of SI retains reactivity with the virus 76K and 31.5K polypeptides as well as with a host cell polypeptide (62h).

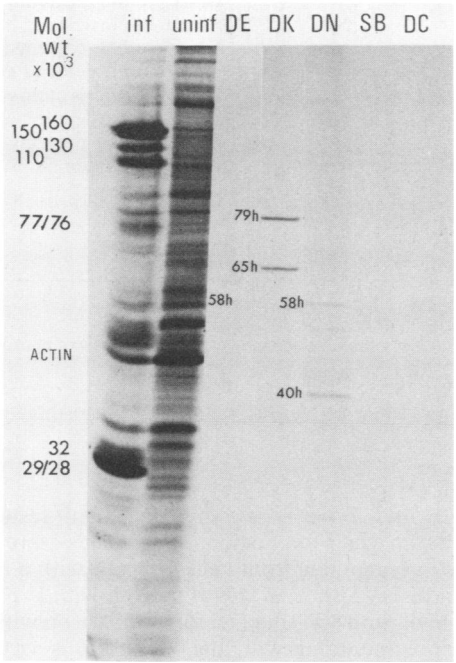

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled polypeptides separated by electrophoresis through 13% polyacrylamide slab gel from immune precipitates formed by the reaction of the DE, DK, DN, SB, and DC monoclonal antibodies with a soluble antigen extract from uninfected cells (track 2). Polypeptides separated from a sample of an extract from infected cells are included as markers (track 1). Major host proteins represented in the immune precipitates are annotated with their apparent molecular weights ($\times 10^3$) and the suffix h (e.g., 79h).

peculiar to this host protein.

Reactions of monoclonal antibodies on Western blots. The polypeptides from lysates of infected cells were separated by electrophoresis on polyacrylamide slab gels, and the separated polypeptides were transferred to sheets of nitrocellulose by transverse electrophoresis. The filters bearing samples of the electroblotted polypeptides were reacted with monoclonal antibodies, and bound antibody was detected with ^{125}I-labeled protein A. Of the eighteen antibodies that were tested, only three reacted with polypeptides after their electrophoretic transfer to nitrocellulose sheets. The SA antibody bound to the 11K polypeptide, the SC antibody bound to the 108K polypeptide, and the SD antibodies bound to both the 65 to 75K and 45 to 52K polypeptides, thus showing the same specificity in direct binding as shown by immune precipitation (data not shown; Table 1).

Kinetic behavior, postsynthetic processing, and strain-specific differences in the electrophoretic mobilities of polypeptides precipitated by monoclonal antibodies. The identity of many of the polypeptides precipitated by monoclonal antibodies could be confirmed on the basis of our previous characterization of polypeptides from infected cells by demonstrating a congruence between the properties of polypeptides immune precipitated and the comigrating species from infected cell extracts. In addition to comigration, three properties were found to be generally useful in achieving these identifications: (i) the kinetic behavior of the polypeptides or, more specifically, measurements of synthesis in the presence and absence of PAA, (ii) the existence of strain-specific differences in electrophoretic mobility, and (iii) the phosphorylation or glycosylation status of the polypeptide.

Immune precipitates were performed with extracts of cells infected with HVS-11Att and labeled with [^{35}S]methionine in the presence of 300 μg of PAA per ml or in the absence of the

TABLE 1. Summary of known properties of monoclonal antibodies to HVS specific polypeptides

Monoclonal antibody	Ability to bind protein A	Neutralization[a] of virus infectivity		Sites of[b] intracellular accumulation	Synthesis in presence of PAA	Polypeptides precipitated[c]	Reaction on[d] western blot	Comments
		Alone	With anti-immunoglobulin					
SA(11K)	+	−	−	Surface	−	11K, 10-11K	+ (11K)	
SB(52K)	+	−	−	Nuclear	+	76K, p50-52K, 31.5K (86K, 40-41K)	−	31.5K, 76K nonstructural delayed early proteins, p50-52K, major immediate early polypeptide
SI(76K)	+	−	−	Not known	+	76K, 31.5K, 62h	−	Nonstructural, delayed early proteins
SC(108K)	+	−	−	Cytoplasmic	−	p108K	+ (108K)	Minor virion phosphoprotein
SD	+	−	+	Cytoplasmic	−	g65-75K, g45-52K	+(65-75K, 45-52K)	65-75K virion glycoproteins
SE	+	(+50%)	+	Cytoplasmic	−	g65-75K, g45-52K	−	65-75K virion glycoproteins
SF(117K)	+	−	−	Cytoplasmic	−	g125-145K, g117K, 47K (g82-92K, g69-76K)	−	g117K precursor to virion glycoproteins
SG(117K)	+	−	−	Cytoplasmic	−	g125-145K, g117K, 47K (g82-92K, g69-76K)	−	g117K precursor to virion glycoproteins
SH(160K)	+	−	−	General	−	160K	−	Virion polypeptide
SJ(160K)	−	−	−	General	−	160K	ND[e]	Virion polypeptide
DI(51K)	±	ND	ND	Nuclear	+	p48-51K	−	Nonstructural, delayed early DNA binding proteins
DC(110K)	+	ND	ND	Nuclear	+	110K	−	Nonstructural, delayed early DNA binding proteins
DD(110K)	+	ND	ND	Nuclear	+	110K	−	Nonstructural, delayed early DNA binding proteins
DF(110K)	+	ND	ND	Nuclear	+	110K	−	Nonstructural, delayed early DNA binding proteins
DJ(110K)	+	ND	ND	Nuclear	+	110K	ND	Nonstructural, delayed early DNA binding proteins
DL(110K)	+	ND	ND	Nuclear	+	110K	ND	Nonstructural, delayed early DNA binding proteins
DM(110K)	+	ND	ND	Nuclear	+	110K	ND	Nonstructural, delayed early DNA binding proteins
DP(110K)	−	ND	ND	Nucler	+	110K	ND	Nonstructural, delayed early DNA binding proteins
DS(110K)	−	ND	ND	Nuclear	+	110K	ND	Nonstructural, delayed early DNA binding proteins
DE(130K)	+	ND	ND	Nuclear	−	130K (58h)	−	Virion polypeptide
DG	+	ND	ND	Not known	+	61K, 59K, (58h)	−	
DB	+	ND	ND	Nuclear	−	Capsid polypeptides	−	
DH	+	ND	ND	Nuclear	−	Capsid polypeptides	−	
DO	+	ND	ND	Nuclear	−	Capsid polypeptides	−	

[a] See Table 2.
[b] As determined by immunofluorescence (see Fig. 8).
[c] Polypeptides are identified by their apparent molecular weight in extracts of cells infected with HVS strain 11. Prominent glycoproteins and phosphoproteins are identified by the prefixes g and p, e.g., g117K, p50 to 52K, etc.
[d] See the text.
[e] ND, Not determined.

inhibitor (19). The labeled polypeptides precipitated by an excess of each monoclonal antibody from the PAA-treated and untreated cultures were separated by gel electrophoresis, and the amount of labeled proteins was analyzed by autoradiography. Polypeptides whose synthesis was relatively sensitive to PAA (late proteins) were clearly differentiated from early (and immediate-early) proteins, the synthesis of which was resistant to PAA (data not shown). In all cases these studies were consistent with the identification of polypeptides on the basis of their comigration with previously recognized early or late proteins (e.g., 48 to 51K, 50 to 52K, 76K, and 110K polypeptides were all PAA resistant, and the 160K, 150K, and 130K polypeptides were PAA sensitive). The results from these experiments are summarized in Table 1.

In Fig. 6 and 7 we show some selected results from experiments in which monoclonal antibodies were used to precipitate polypeptides from cells infected with a heterologous strain of HVS (i.e., KM744) labeled with [^{35}S]methionine (Fig. 6) or with $^{32}P_i$ (Fig. 7), for comparison with results of similar precipitations with the homologous virus strain (HVS-11Att; Fig. 2 and 3 for [^{35}S]methionine-labeled proteins and Fig. 7 for $^{32}P_i$-labeled proteins). No examples of strain-specific epitopes have been noted. The monoclonal antibodies raised against HVS-11Att precipitated single (Fig. 6; SA, 11K; DC, 110K) or multiple (Fig. 6; SE, 45 to 52K and 65 to 75K; DB, capsid polypeptides) polypeptides from extracts of cells infected with heterologous virus similar to those precipitated from cells infected with the homologous virus strain (Fig. 2 and 3). Moreover, in those cases where

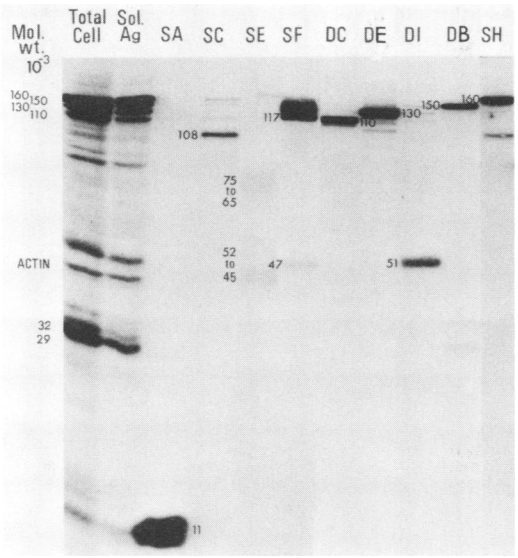

FIG. 6. Autoradiogram of [^{35}S]methionine-labeled polypeptides from a lysate of OMK cells infected with HVS strain KM744 (track 1) and the immune precipitates formed by the reaction of monoclonal antibodies (SA, SC, SE, SF, DC, DE, DI, DB, SH) with a soluble antigen extract from these cells (track 2), separated by electrophoresis through a 13% polyacrylamide gel. Prominent virus-specific polypeptides precipitated by these monoclonal antibodies are annotated with the apparent molecular weights ($\times 10^3$) of the homologous polypeptides from HVS-11Att-infected cells (see the text for the system of nomenclature). However, there are strain-specific differences in the apparent molecular weights of some polypeptides (e.g., the 108K polypeptide precipitated by SC antibody from extracts of cells infected with strain KM744 has a lower apparent molecular weight than the homologous polypeptide of HVS-11Att; see Fig. 7).

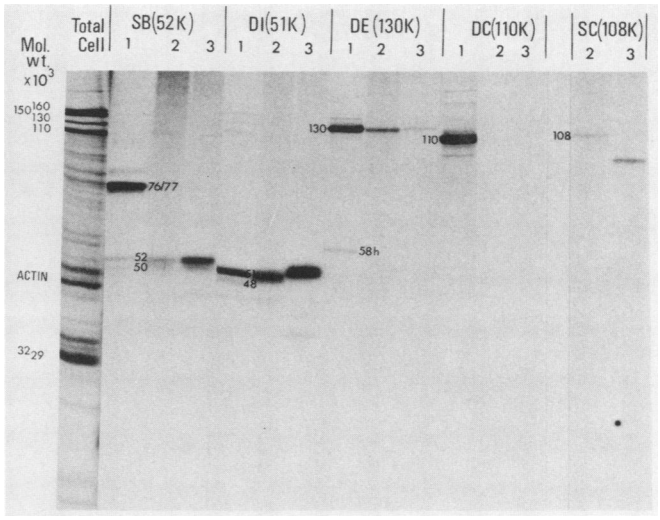

FIG. 7. Autoradiogram of labeled proteins and phosphoproteins separated by electrophoresis through a 12% polyacrylamide slab gel from immune precipitates formed by reacting monoclonal antibodies SB(52K), DI(51K), DE(130K), DC(110K), and SC(108K) with the following antigen extracts: (tracks 1) cells infected with HVS-11Att and labeled with [^{35}S]methionine from 24 to 72 h after infection, (tracks 2) cells infected with HVS-11Att and labeled with $^{32}P_i$ from 24 to 72 h after infection, and (tracks 3) cells infected with HVS-KM744 and labeled with $^{32}P_i$ from 24 to 72 h after infection. Polypeptides separated from a sample of the total lysate of cells infected with HVS-11Att and labeled with [^{35}S]methionine (total cell, leftmost track) were included as molecular weight markers, and polypeptides are annotated as in the preceding figures.

the polypeptides precipitated from HVS-11Att were glycosylated or phosphorylated the polypeptides precipitated from cells infected with HVS strain KM744 were also glycosylated or phosphorylated. For example, the 125 to 145K and 117K polypeptides precipitated by antibody SF from both strains are glycosylated (data not shown), and the 50 to 52K polypeptides precipitated by SB and the 48 to 51K polypeptides precipitated by DI are phosphoproteins in extracts of cells infected with each of these virus strains (Fig. 7). There are small strain-specific differences in the electrophoretic mobilities of many of the homologous proteins (48 to 51K, Fig. 7), and some polypeptides show more marked strain-specific differences in their apparent molecular weight (e.g., the 108K protein precipitated from HVS-11Att-infected cells by antibody SC has a homolog with a lower apparent molecular weight in cells infected with HVS strain KM744). In the case of the 108K phosphoprotein (Fig. 7), these strain differences were useful in confirming its identity with a minor virion phosphoprotein, which shows the same pattern of strain variation in apparent molecular weight (data not shown). The mobility of this polypeptide on gels of different acrylamide concentration is also markedly anomalous relative to the 150K, 130K, and 110K proteins (on lower gel strength [10%], it comigrates with the 130K polypeptide from HVS-11Att; data not shown). The results shown in Fig. 7 also confirm that the 50 to 52K polypeptides precipitated by antibody SB are phosphoproteins, whereas the coprecipitated 76K polypeptide is not, and that the 130K is detectably phosphorylated and the 110K polypeptide is not.

Site of intracellular accumulation of virus antigens as demonstrated by immunofluorescence staining of infected cells with monoclonal antibodies. Figure 8 demonstrates the distinctive patterns of fluorescence obtained when HVS-infected cells were stained with monoclonal antibodies to different virus antigens. Thus, antibodies SB(52K), DC(110K), DE(130K), DH(capsid), and DI(51K) selectively stain the nuclei of infected cells. However, the pattern of the subnuclear fluorescence was characteristic for each of these antibodies and independent of the uniform distribution of cellular DNA monitored by simultaneous fluorescence microscopy of the same cells stained with DAPI (Fig. 8; DAPI-SB, DAPI-SH). The antigen recognized by SB(52K) antibody was relatively evenly distributed throughout the nuclear volume at all times during infection. The DI(51K) antibody gave more localized nuclear fluorescence, especially at late times after infection when large, intensely fluorescent, aggregates were seen. In contrast, antibodies to the 110K DNA-binding protein, e.g., DC(110K), stained the nuclei at very many granular foci, giving rise to very distinctive punctate fluorescence. The reaction of antibody to the capsid polypeptides [DH(capsid)] was located at or near the inner surface of the nuclear membrane at early times in infection (Fig. 8, arrows), whereas at later times large accumulations of antigen within the nucleus and some cytoplasmic fluorescence could be visualized. Staining infected cells with antibody (DE) to the 130K structural protein resulted in diffuse nuclear fluorescence and some cytoplasmic fluorescence at later times in infection. Antibody to the 108K minor structural phosphoprotein (SC) gave only granular cytoplasmic fluorescence, and antibodies to the virus glycoproteins (e.g., SD and SF) gave more diffuse

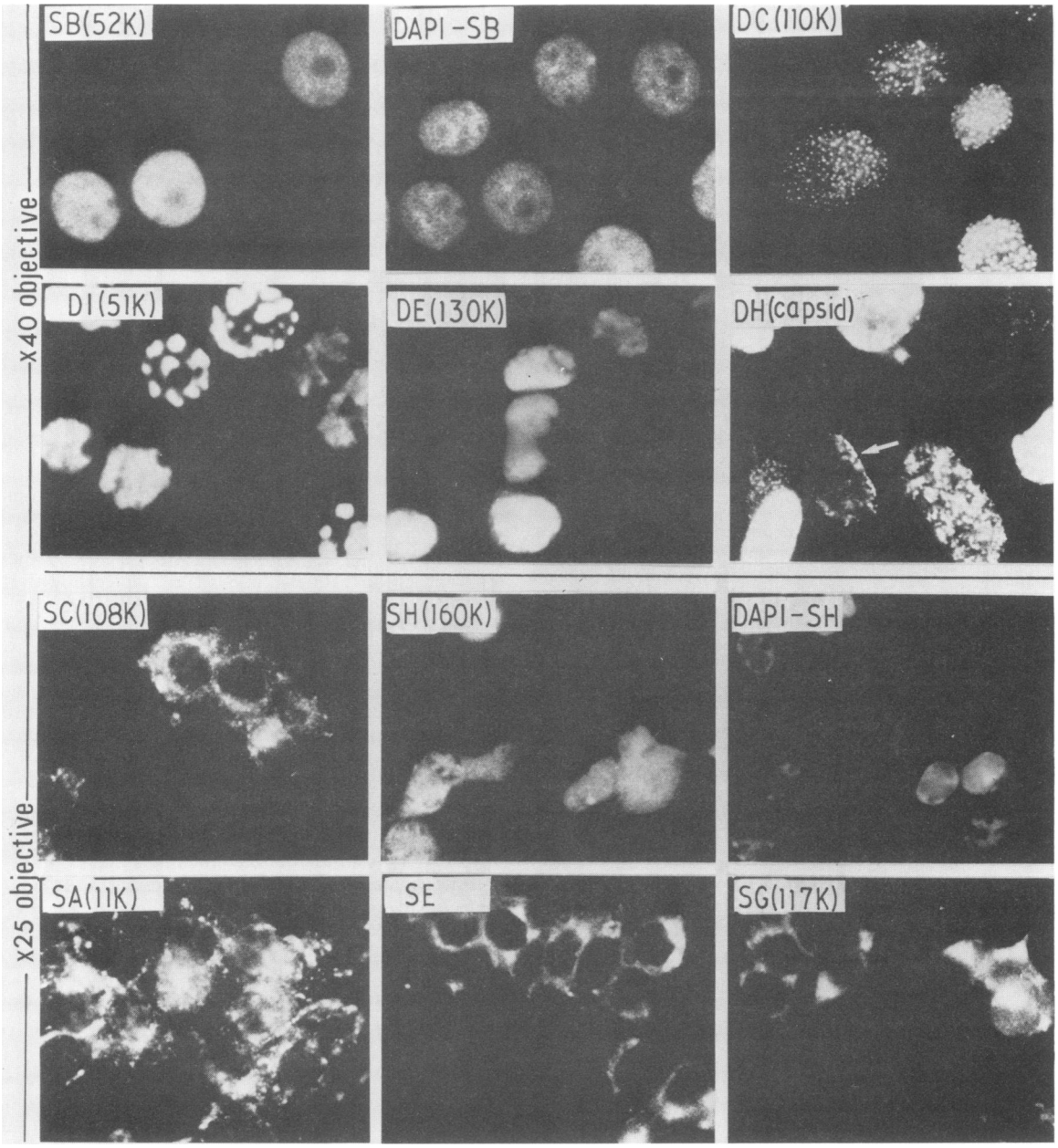

FIG. 8. Photomicrographs of the intracellular distributions of virus antigens in infected cells which react with monoclonal antibodies SB(52K), DC(110K), DI(51K), DH(capsid), DE(130K), SH(160K), SC(108K), SG(117K), SE, and SA(11K) as visualized by indirect immunofluorescence with rhodamine-conjugated goat anti-mouse immunoglobulin. All cells were also counterstained with the DNA-binding fluorochrome, DAPI, to allow the comparison of the distribution of cellular DNA with the distribution of virus antigens. Panels DAPI-SB and DAPI-SH show DAPI fluorescence in the same cells for which rhodamine fluorescence is illustrated in panels SB(52K) and SH(160K). The micrographs in the upper six panels were taken with a ×40 objective lens, and those in the lower six panels were taken with a ×25 objective lens.

cytoplasmic fluorescence as well as some surface fluorescence. The SA(11K) antibody also gave some cytoplasmic fluorescence, but the most distinctive feature of the reaction of this antibody was an intense surface fluorescence at late times after infection. Antibody SH(160K) gave general fluorescence of both cytoplasm and nuclei. No clear pattern of fluorescence was observed when infected or uninfected cells were stained with monoclonal antibodies SI(76K), DG, DA, or DR. Antibody DN, which precipitated the 79K and 65K host polypeptides, stained all of the nuclei of infected and uninfected cells.

Neutralization of virus infectivity. The above experiments identified a number of monoclonal antibodies that reacted with glycosylated or nonglycosylated proteins that were known to be components of the virus particle, but not part of the structure of the virus capsid shell (2, 10, 21; unpublished results). These and control antibodies (SB) to the nonstructural 50 to 52K polypeptides were tested for their ability to neutralize virus directly and for their ability to sensitize virus to subsequent neutralization by anti-mouse antibodies in a simple plaque reduction test. The results are summarized in Table 2. None of the monoclonal antibodies neutralized the

TABLE 2. Neutralization of virus infectivity by monoclonal antibodies in the presence and absence of anti-mouse immunoglobulin

Monoclonal antibody	% Surviving infectivity[a] at the following dilutions of monoclonal antibodies[b]:					
	With calf serum			With anti-mouse immunoglobulin[c]		
	10^{-1}	10^{-3}	10^{-6}	10^{-1}	10^{-3}	10^{-6}
SA(11K)	96	119	ND[d]	107	93	ND
SB(52K)	97	105	ND	110	100	ND
SC(108K)	126	80	ND	72	78	ND
SD	106	98	ND	2	2	65
SE	47	54	53	1	1	2
SF(117K)	96	115	91	58	86	112
SG(117K)	103	81	111	120	78	98
SH(160K)	109	120	112	76	75	91
SJ(160K)	102	111	ND	86	82	103

[a] Indicator virus was HVS-11Att, and 100% survival was 2,500 PFU/ml. Results are means from three experiments.
[b] Relative dilutions of ascitic fluids.
[c] One-fifth dilution of sheep anti-mouse immunoglobulin.
[d] ND, Not determined.

majority of the infectious virus population directly. Antibody SE neutralized about 50% of the virus population even at very high dilutions, and antibodies SE and SD sensitized 98% of the infectious virus for subsequent neutralization by anti-mouse immunoglobulin. Both of these monoclonal antibodies precipitate the 62 to 75K and 45 to 52K glycoproteins from extracts of infected cells (Fig. 2) and precipitated 62 to 75K glycoproteins from detergent extracts of purified enveloped virus particles. None of the other antibodies sensitized virus to subsequent neutralization by anti-mouse immunoglobulin (Table 2). Since the SF and SG antibodies and the SH and SJ antibodies also precipitate polypeptides from detergent extracts of virus particles (results not shown), it appears that the relevant epitopes are not available for antibody binding in the intact infectious virus particle.

DISCUSSION

In this paper we have applied an improved screening procedure to isolate monoclonal antibodies against structural and nonstructural proteins of HVS. We also present data on the characterization of these antibodies and some of the properties of virus proteins which have been revealed by experiments exploiting these reagents.

A specific, sensitive, and rapid screening procedure to identify those clones secreting antibodies of the required specificity and eliminate unnecessary manipulations with negative clones is a primary requirement for an effective program of monoclonal antibody isolation. The method we have applied fulfills these requirements; it uses an inexpensive source of all virus antigens bound at relatively high concentrations to nitrocellulose. It is also capable of testing relatively large volumes of culture medium and is thus capable of detecting low antibody concentrations. The facility with which the method discriminated between protein A-binding and nonbinding classes of mouse immunoglobulins was also a considerable practical advantage. After the detection of clones secreting antibody specific to virus antigens, we chose to concentrate on those that bound protein A because of the experimental convenience of this latter property. By applying this screening procedure we have isolated antibodies to more than 13 different virus-specific polypeptides, and a number of these proteins are normally present at very low concentrations in extracts from infected cells [e.g., SC(108K)]. The present method does not select on any basis other than the capacity to bind antigen on a solid support; selection for a particular biological effect (e.g., neutralization) would have detected only a very small subset of the present antibodies.

Antibodies to the immediate-early 52K polypeptide (SB) and to the 76K delayed-early polypeptide (SI) were each isolated from fusions by using splenocytes from mice immunized with purified virus. We cannot detect either of these polypeptides in preparations of purified virions, and neither of these monoclonal antibodies reacts in immune precipitation with detergent extracts from purified virions. However, we have shown that HVS undergoes an abortive cycle of replication in mouse cells in culture and that this abortive cycle is characterized by high rates of immediate-early and early protein synthesis and markedly reduced rates of late protein synthesis (unpublished results). Similar abortive infections of mouse cells have been reported with a simian cytomegalovirus (9). These results suggest that an abortive infection of cells in the immunized mice was the source of these early antigens. Such abortive infections may provide a useful means for selective immunization against nonstructural early proteins.

The specificities of many of the antibodies we have isolated are uncomplicated and provide reagents to investigate many of the functionally distinct proteins specified by HVS (Table 1). The present studies on the intracellular distributions of virus proteins provide a simple example of the information that these reagents can provide. The intracellular distributions of the 110K and 51K DNA-binding proteins differ from each other in a characteristic way, and in neither case does their distribution resemble that of cellular DNA. The highly localized concentrations of the 110K protein may reflect its presence at specific, functionally significant sites. Others of the antibodies precipitate multiple polypeptides; in the cases of the DB, DH, and DO monoclonal antibodies the polypeptides precipitated include polypeptides of the virus capsid. None of these antibodies reacted in Western blots, and thus we cannot identify their binding sites unequivocally, but in each case they appear to precipitate preexisting complexes of antigenically unrelated polypeptides. The polypeptides precipitated by DB antibody are identical in number and molarity to those of a population of capsids isolated by equilibrium or velocity gradient sedimentation of nuclear extracts from infected cells (2; unpublished results). Related, but nonidentical, complexes are precipitated by the DH and DO antibodies. The results of our immunofluorescence studies indicate that accumulations of antigens reactive with these anti-capsid antibodies are first detected at the periphery of the nucleus, and these reagents should be invaluable for studies of the assembly of virus capsids. Likewise, antibodies to the virus glycoproteins will be useful for studies of their postsynthetic processing and complex formation involving these polypeptides. Results from the Western blotting experiments with antibody SD show that the 65 to 75K and 45 to 52K glycoproteins share antigenic determinants and are likely to be related. The multiple glycoproteins precipitated by the SF and SG antibodies may also be processed forms of a single precursor. However, a nonglycosylated 47K polypeptide is a reproducible and specific component of immune precipitations with these antibodies, and further experiments will be necessary to discriminate between the presence of a common antigenic site and the formation of a stable complex of related or

unrelated polypeptides. Examples of relatively unstable complexes between unrelated polypeptides have been observed with the 160K and 108K polypeptides (Fig. 6, antibodies SC and SH) even under our relatively rigorous conditions for immune precipitation (i.e., 0.65 M NaCl, 1% Nonidet P-40) and under less stringent conditions (i.e., 0.15 M NaCl, 1% Nonidet P-40) the SC(108K) antibody precipitates a complex of virion-related polypeptides including noncapsid as well as capsid polypeptides (unpublished results).

Two other results require some further comment; the specific precipitation of the 62K host protein by monoclonal antibody SI(76K) and the precipitation of both the virus immediate-early 52K phosphoprotein and the nonphosphorylated 76K delayed-early protein by antibody SB. Neither of these results was attributable to complex formation (see above), and we must therefore accept the likelihood that a virus and a host protein share the SI epitope and that two differently regulated virus proteins share the epitope recognized by antibody SB. There are precedents for both of these situations. In the case of reactions with host proteins not known to be related by any extensive homology of sequence (14), such reactions have been attributed to coincidental occurrence of a common antigenic site on otherwise unrelated proteins which may or may not have some relevance to a shared biological function (3). In the case of the two virus gene products, a simple probabilistic argument is less convincing, and interpretations that suggest limited regions of true homology derived from common coding sequences are more attractive. There are precedents for the synthesis of distinct proteins with some common amino acid sequences as the result of differential splicing or promoter usage (5, 6). In the present case we know that the virus gene products are members of differently regulated classes, but we do not have the necessary information on the location or structure of their coding sequences to be able to differentiate these explanations from alternatives such as the common possession of a simple repeating motif of amino acids originating from widely separated genes. The occurrence of repeated sequences of nucleotides within coding sequences has been noted in other herpesviruses (Epstein-Barr virus [7] and herpes simplex virus [24]), and the presence of such sequences clearly increases the probability of a common antigenic site on otherwise unrelated proteins.

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