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Varicella-Zoster Viral Glycoproteins Analyzed with Monoclonal Antibodies

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Monoclonal antibodies to varicella-zoster virus were used to study viral glycoproteins by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Based on the viral glycoproteins immunoprecipitated, the five monoclonal antibodies fell into three groups. Two antibodies, 4B7 and 8G9 (group 1), immunoprecipitated a single glycoprotein of molecular weight (MW) 118,000 (118K glycoprotein) and had high neutralizing activity in the absence of complement. One antibody, 3C7 (group 2), which lacked neutralizing activity, immunoprecipitated two glycoproteins of MWs 120,000 and 118,000 and a glycoprotein giving a diffuse band in the region of 64,000 to 65,000. Pulse-chase experiments and experiments with monensin as an inhibitor of glycosylation suggested that the 120K polypeptide was derived by glycosylation of the 118K polypeptide and that a 43K antigen was processed into the 64 to 65K glycoprotein. Two antibodies, 3G8 and 4E6 (group 3), both had neutralizing activity only in the presence of complement, and both immunoprecipitated at least five polypeptides, with MWs ranging from 50,000 to 90,000. Antibody 3G8 was isotype immunoglobulin G2b (IgG2b), and its immunoprecipitating activity was stronger than that of 4E6, which was isotype IgG1. Pulse-chase experiments with antibody 3G8 showed that lower-MW glycopeptides chased into three polypeptides of MWs 90,000, 80,000, and 60,000 by 24 h. Immunoprecipitation experiments with antibody 3G8 on infected cells treated with glycosylation inhibitors 2-deoxyglucose, monensin, and tunicamycin, suggested that a prominent, early-appearing 70K polypeptide may have been processed into the glycoproteins of higher MWs and that the 60K polypeptide may have been derived by glycosylation of polypeptides of lower MWs.

Several reports have appeared in recent years on the characterization of the polypeptides of varicella-zoster virus (VZV), and the results have varied somewhat. This may be related in part to the difficulties inherent in growing and purifying the virus, because of its strongly cell-associated nature, and to problems encountered in producing immune reagents for antigenic analysis which are free from antibodies to host proteins. In studies in which sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare proteins from lysates of VZV-infected cells with those from uninfected cells, 16 to 33 polypeptides were described (1, 2). Other studies in which purified virions were used for VZV polypeptide analysis by SDS-PAGE also identified up to 33 polypeptides (24, 25, 28). VZV antisera produced in monkeys, guinea pigs, and rabbits, and also sera from human VZV infections, have been used to study the viral polypeptides by immunoprecipitation, and up to 33 polypeptides, including five to nine glycosylated ones, have been reported (1, 10, 12, 29). The polyclonal antisera could not, however, provide information on the immunological specificity of individual polypeptides or on the relationship of the individual polypeptides to one another. More recently, Grose et al. (11) and Okuno et al. (18) have produced monoclonal antibodies to VZV and used them to study the viral glycoproteins by immunoprecipitation and SDS-PAGE.

A panel of several independently derived monoclonal antibodies to VZV has been produced in this laboratory, and their serological activity in several different antibody assay systems has been described (8). In the present study, we identified the viral polypeptides immunoprecipitated by these monoclonal antibodies and used the antibodies to study the synthesis and processing of viral glycoproteins.

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MATERIALS AND METHODS

Virus. The CaQu strain of VZV, an isolate from this laboratory, was propagated in a line of human fetal diploid lung (HFDL) cells established by J. H. Schieble of this laboratory. Cell-free virus with titers $\geq 10^6$ PFU/ml, produced as previously described (22), was used throughout the studies.

Serological assays. The neutralization (21), indirect immunofluorescence staining (5), and enzyme immunofluorescence (6, 7) assays for VZV antibodies have been described previously.

Production of VZV monoclonal antibodies. The methods employed for producing hybridomas secreting VZV antibodies have been described in detail previously (8). Briefly, BALB/c mice were immunized with sucrose density gradient-purified virions and nucleocapsids. After 4 weeks, their spleen cells were fused with mouse myeloma cells of the SP 2/0 Ag-14 line. Hybrid cells were selected in hypoxanthine-aminopterin-thymidine medium, and VZV antibody-producing clones were identified by enzyme immunofluorescence, indirect immunofluorescence staining, and neutralization tests. High-titer mouse ascitic fluids were produced by injecting antibody-producing clones intraperitoneally into pristane-primed mice.

Radioisotope labeling of VZV. Monolayers of HFDL cells in T-75 plastic flasks were infected with cell-free VZV at a ratio of 1 PFU per cell. At 48 h postinfection, the medium was removed, and the cultures were rinsed with the deficient medium to be used for labeling and then held for 1 to 2 h on medium consisting of 1% dialyzed fetal bovine serum and Eagle minimal essential medium with 10% of the normal concentration of methionine or glucose, depending on the radiolabel to be used. The infected cells were then labeled with either 20 μCi of [^{35}S]methionine per ml or 2 μCi of D-[^{14}C]glucosamine hydrochloride (New England Nuclear Corp., Boston, Mass.) per ml for 16 to 20 h. The labeled

medium was then removed, and the monolayers were rinsed three or four times with 0.01 M phosphate-buffered saline (PBS), pH 7.4. Ice-cold PBS containing 1% deoxycholate, 1% Nonidet P-40, and 1% aprotinin (to block protease activity released from the cells) was added to the cells. The cells were dislodged into the buffer, transferred to a 10-ml polycarbonate tube, and agitated vigorously on a Vortex apparatus for 20 s at 3- to 4-min intervals for 30 min. The cells were then frozen and thawed twice and sonicated for 30 s. Finally, the cell lysate was centrifuged at 40,000 × *g* for 1 h, and the supernatant fluid was removed carefully and stored at −70°C. Uninfected cells of the same lot were labeled and processed in the same manner for use as a control. Radiolabeled preparations were dissolved in Aquassure scintillation cocktail (New England Nuclear), and the radioactivity was monitored in a Beckman beta-counter.

Radioimmunoprecipitation assays. A 0.5-ml sample of the cell culture medium from antibody-producing hybridoma cultures or 4 to 5 μl of mouse immune ascitic fluid were mixed with 10^6 cpm [^{35}S]methionine-labeled or 10^5 cpm of [^{14}C]glucosamine-labeled VZV antigen (40 to 50 μl). The mixtures were incubated for 1 h at 37°C and then overnight at 4°C. Goat anti-mouse gamma globulin (Antibodies, Inc., Davis, Calif.) was added to preswollen staphylococcal protein A-Sepharose beads (Sigma Chemical Co., St. Louis, Mo.) in PBS containing 1% deoxycholate, 1% Nonidet P-40, and 1% aprotinin at a ratio of 2 μl of serum to 1 mg of protein A-Sepharose beads. Absorption proceeded for 2 h at room temperature with occasional mixing. The beads were then washed three times in the above buffer to remove unbound antibody.

The antibody-treated Sepharose beads (5 mg) were added to the labeled antigen-antibody mixtures and incubated for 2 h at room temperature with gentle mixing. The mixtures were then centrifuged at 800 × *g* for 10 min, and the supernatant fluids were aspirated and discarded. The beads were washed four to five times with PBS containing 1% deoxycholate, 1% Nonidet P-40, and 1% aprotinin, and then 0.1 ml of disrupting buffer (0.125 M Tris-hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.002% bromophenol blue) was added to the washed beads, and they were heated in boiling water for 3 min.

Slab gel electrophoresis. SDS-PAGE was performed by the method of Laemmli (15). Stock solutions of acrylamide and *N,N-bis*-methyleneacrylamide at a ratio of 30:0.8 were polymerized with tetramethylethylenediamine and ammonium persulfate in Tris-hydrochloride buffer, pH 8.8, as recommended by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Separation gels were cast with 9 to 10% acrylamide and held at 4°C for 20 h before they were used. The radiolabeled samples were added to wells in stacking gel cast with 3 to 4% acrylamide in Tris-hydrochloride buffer, pH 6.8. The slab gels were 14 cm wide, 32 cm long, and 1.5 mm thick.

Electrophoresis was performed on a Bio-Rad Protean vertical slab gel electrophoresis unit. The electrode buffer was 0.025 M Tris, 0.192 M glycine, and 0.1% SDS, pH 8.3. Samples of disrupted VZV antigen or immunoprecipitates were electrophoresed at a constant current of 13 to 14 mA per gel for 16 to 18 h, or until the tracking dye reached the bottom of the gel. After electrophoresis, the gels were fixed in 40% methanol–10% acetic acid and stained with Coomassie blue. After destaining, the gels were transferred into a fluor solution (En^{3}Hance; New England Nuclear) for 1 h by the method of Bonner and Laskey (3). The gels were then rinsed several times in distilled water, transferred to filter paper, and dried with a vacuum-heated gel drier (Bio-Rad). Fluorographs of the dried gels were made on Kodak XRP-1 film exposed at −70°C.

The molecular weights (MWs) of the polypeptides were estimated by running a ^{14}C-labeled marker protein kit (Bethesda Research Laboratories, Gaithersburg, Md.) on the same gel. The protein markers were myosin (H chain) (200,000 MW [200K]), phosphorylase b (92.5K), bovine serum albumin (68K), ovalbumin (43K), alpha-chymotrypsinogen (25.7K), beta-lactoglobulin (18.4K), and cytochrome *c* (12.3K). For comparison, unlabeled molecular markers (Bio-Rad) were used and stained with Coomassie blue.

Study of the kinetics of viral protein synthesis. HFDL cell cultures infected with cell-free VZV at a ratio of 1 PFU per cell were radiolabeled with 20 μCi of [^{35}S]methionine per ml or 2 μCi of [^{14}C]glucosamine per ml from 2 to 8, 8 to 16, 16 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, or 120 to 144 h postinfection. Cell lysates were prepared as described above for use in immunoprecipitation and SDS-PAGE.

Pulse-chase labeling. The optimal pulse-labeling time interval for pulse-chase experiments was determined by labeling cultures showing a viral cytopathic effect in 50 to 60% of the cells at 48 to 72 h postinfection with 50 μCi of [^{35}S]methionine per ml for 10, 20, 30, 40, 50, 60, 75, or 90 min. For the final pulse-chase experiments, cultures at 48 h postinfection were pulse-labeled for 10 to 15 min and chased for 3 or 24 h.

Studies with inhibitors of glycosylation. HFDL monolayer cell cultures were infected with cell-free VZV at a ratio of 1 PFU per cell, and at 24 h postinfection the culture medium was replaced with Eagle minimal essential medium and 5% fetal bovine serum containing either 2-deoxyglucose (10 mM), monensin (0.1%), or tunicamycin (3 μg/ml). Earlier addition of inhibitors, especially 2-deoxyglucose, inhibited viral infection and protein synthesis. After 24 h of incubation, the cells were labeled for 20 h with 20 μCi of [^{35}S]methionine per ml in methionine-deficient medium containing the same glycosylation inhibitor. The inhibitors were present during the entire labeling time, and lysates of the cells were prepared as described above. The concentrations of inhibitors used were those reported to be optimal for inhibition of herpes simplex virus glycosylation (9, 13, 17).

RESULTS

Development of VZV polypeptides in infected cells. To obtain preliminary information on the synthesis of viral polypeptides in cells infected with cell-free virus, the infected cultures and uninfected control cultures were labeled at various times postinfection with [^{35}S]methionine or [^{14}C]glucosamine and examined by SDS-PAGE as described above. Most previous studies have used VZV-infected cells as viral inocula, and it was thought that the use of cell-free virus might give a better-synchronized infection and might also facilitate the identification of virus-specific early antigens. Preliminary studies with [^{35}S]methionine labeling indicated that viral antigen could not be detected until 16 h postinfection, although cellular antigens showed heavy labeling, which might have masked low levels of virus-specific antigen. Immunofluorescence staining and infectious center assays indicated that approximately 100% of the cells were infected at 16 h. At 16 to 24 h postinfection, at least 13 viral polypeptides could be identified which were not present in uninfected cultures; the production of these antigens increased, and maximum levels were detected at 48 to 72 h. At this time, 18 virus-specific proteins were detected with MWs ranging from 150,000 to 22,000. The most prominent ones were seen at 150K, 120K, 90K, 80K, 70K, 65K, and 60K.

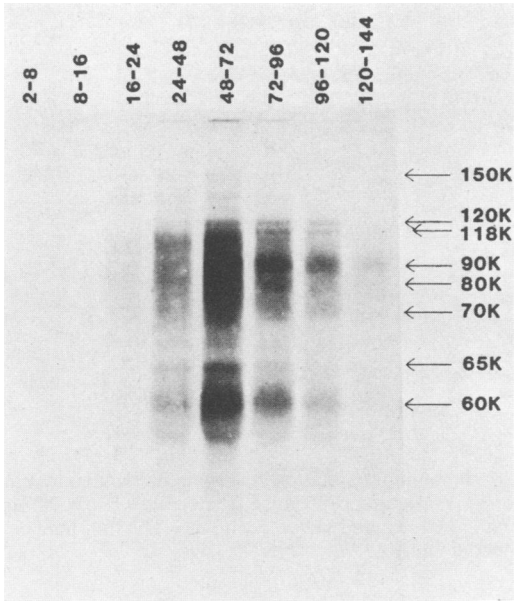

FIG. 1. Immunoprecipitation reactions of polyclonal VZV-immune monkey serum with infected cells labeled with [^{14}C]glucosamine at the time intervals (hours) postinfection indicated above each lane.

With further incubation of the infected cultures, the production of viral antigen decreased, and by 120 to 144 h most of the antigen had disappeared from the cells (data not shown).

When [^{14}C]glucosamine was used to detect viral glycoproteins, only three labeled proteins, with MWs of 90,000, 80,000, and 65,000, were detected at 16 to 24 h postinfection. At 48 to 72 h, 14 labeled bands were detected, the most prominent being at 150K, 120K, 90K, 80K, 70K, 65K, and 60K. The virus specificity of these antigens was evidenced by their absence in uninfected control cultures. Many of the labeled bands probably represented precursors or products of the major viral glycopeptides in various stages of glycosylation, which would impart a wide variety of MWs to the glycopeptides. At later labeling times, most of the antigens had disappeared from the cells (data not shown).

The MWs of polypeptides detected in our time course studies with cell-free virus inocula were similar to those detected by other workers who used infected cells as inocula (1, 2, 12).

Viral polypeptides immunoprecipitated with polyclonal VZV antiserum. To provide a baseline for immunoprecipitation studies with monoclonal antibodies, viral proteins were precipitated from VZV-infected cells with hyperimmune antiserum produced in a rhesus monkey (23); preimmunization serum was used as a control. Based on the time course studies described above, the cells were radiolabeled from 48 to 72 h postinfection. With infected cells labeled with

TABLE 1. Reactivity of VZV monoclonal antibodies

Group	Neutralizing activity	Clone	Isotype	Glycoproteins immunoprecipitated
1	Complement independent	4B7	IgG1	118K
		8G9	IgG1	
2	None	3C7	IgG1	120K, 118K, 64-65K
3	Complement dependent	3G8	IgG2b	90K, 80K, 70K, 65K,
		4E6	IgG1	60K, 50K

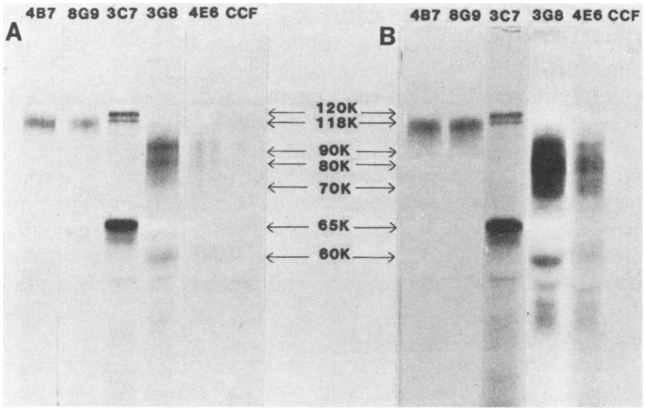

FIG. 2. VZV glycopeptides immunoprecipitated with various monoclonal antibodies (cell culture fluids). At 48 h postinfection, cells were labeled with [^{35}S]methionine (A) or [^{14}C]glucosamine (B) for 18 h. CCF, Control mouse myeloma cell culture fluid.

[^{35}S]methionine, the hyperimmune monkey serum precipitated at least 16 polypeptides which were not precipitated with preimmunization serum; these had MWs ranging from 200,000 to 30,000. With [^{14}C]glucosamine-labeled infected-cell lysates, 12 virus-specific glycoprotein bands were precipitated, with MWs ranging from 155,000 to 40,000. The most prominent glycoprotein bands were detected at 118K to 120K, 80K to 90K, and 60K to 65K. It is likely that some of the multiple glycoprotein bands represented precursors or products in various stages of glycosylation. Similar polypeptide and glycoprotein profiles were reported by investigators who used polyclonal VZV antisera from other sources for their immunoprecipitation studies (1, 11). A faint band detected at 150K with [^{14}C]glucosamine labeling would appear to correspond to the high-MW major capsid protein described by other workers (1, 12, 29).

The kinetics of synthesis of glycoproteins which could be precipitated by the polyclonal VZV antiserum was then studied in cells labeled at various times postinfection. In cells labeled with [^{14}C]glucosamine, the first antigen was detectable in trace amounts at 8 to 16 h postinfection and had an MW of 90,000 (Fig. 1). This antigen reached maximum levels in the interval from 48 to 72 h postinfection and then

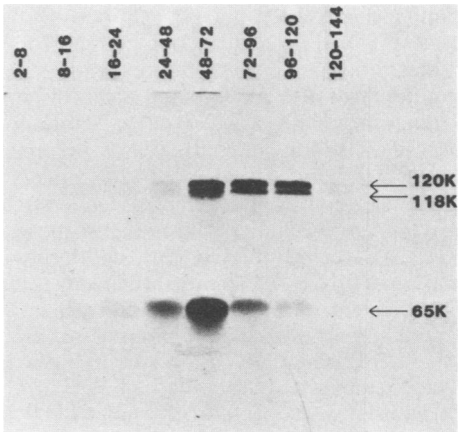

FIG. 3. Immunoprecipitation reactions of monoclonal antibody 3C7 with VZV-infected cells labeled with [^{14}C]glucosamine at the time intervals (hours) postinfection indicated above each lane.

decreased with longer incubation. Similar results were obtained with [^{35}S]methionine-labeled infected cells (data not shown).

VZV polypeptides precipitated by monoclonal antibodies. When infected-cell lysates labeled with [^{35}S]methionine or [^{14}C]glucosamine were immunoprecipitated with VZV monoclonal antibodies and analyzed by SDS-PAGE, some of the monoclonal antibodies were seen to immunoprecipitate a single polypeptide, whereas others coprecipitated more than one polypeptide. Based on their biological properties and the viral polypeptides which they immunoprecipitated, the five monoclonal antibodies were seen to fall into three groups (Table 1 and Fig. 2).

Several possibilities were considered to explain the reactivity of a monoclonal antibody with more than one polypeptide. First, the possibility that the antibody-producing cells had not been cloned properly and consisted of multiple hybrids was ruled out by recloning four times and reexamining the antibodies by immunoprecipitation. Identical results were obtained with the recloned hybridomas. This led to the consideration that the antibodies may have been reacting with an epitope common to several polypeptides. Some of the polypeptides may be precursors or cleavage products of the others, or they may exist in different forms of glycosylation, which would cause them to have different MWs. Various experiments were conducted in efforts to elucidate why some of the monoclonal antibodies reacted with more than one polypeptide or glycoprotein; these are described below.

Group 1 monoclonal antibodies. The two group 1 antibodies, 4B7 and 8G9, had the immunoglobulin G1 (IgG1) isotype of mouse immunoglobulins; they neutralized VZV to high titer, and their neutralizing activity was independent of complement (7). When [^{35}S]methionine- or [^{14}C]glucosamine-labeled VZV infected-cell lysates were immunoprecipitated with these antibodies and subjected to SDS-PAGE, a single glycopeptide with an MW of ca. 118,000 was precipitated (Fig. 2). In studies on the kinetics of synthesis of this glycopeptide, it was first detected at 16 to 24 h postinfection, reached maximum levels at 48 to 72 h, and then disappeared rapidly (data not shown). It would appear that both of the antibodies are directed against epitopes on the same glycoprotein which are important for virus neutralization. Competition binding assays will be required to determine whether the antibodies are directed against the same epitope on the glycoprotein.

Group 2 monoclonal antibody 3C7. Antibody 3C7 was also of isotype IgG1; it had no neutralizing activity for VZV and gave a granular indirect immunofluorescence staining pattern in the cytoplasm of VZV-infected cells. In immunoprecipitation reactions with lysates of [^{35}S]methionine- or [^{14}C]glucosamine-labeled infected cells, it precipitated two polypeptides which banded sharply at 120K and 118K and a large diffuse band at 64K to 65K which extended to 62K (Fig. 2). To study the synthesis and processing of these proteins, we labeled HFDL cells infected with cell-free virus with [^{35}S]methionine or [^{14}C]glucosamine at various times postinfection. The cell lysates were then reacted with antibody 3C7 cell culture medium or with mouse immune ascitic fluid and subjected to SDS-PAGE (Fig. 3). At 8 to 16 h postinfection, the first glycoprotein precipitated had an MW of 118,000; at 16 to 24 h another glycoprotein of MW 64,000 to 65,000 appeared; and at 24 to 48 h another antigen emerged with an MW of 120,000. All these antigens were present at maximum concentrations at 48 to 72 h postinfection and diminished as the infection progressed.

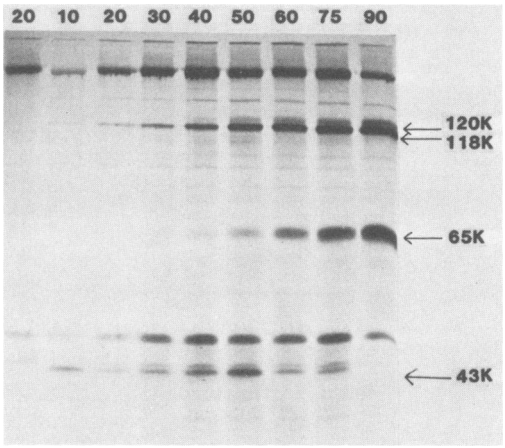

FIG. 4. Immunoprecipitation reactions of monoclonal antibody 3C7 with VZV-infected cells pulse labeled with [^{35}S]methionine for 10 to 90 min as indicated above each lane. The first lane contained an uninfected control.

To determine the interrelationships among the 120K, 118K and 64–65K glycoproteins, cells at 48 h postinfection were pulse-labeled for 10 to 90 min as described above. The lysates were immunoprecipitated with cell culture fluid or mouse immune ascitic fluid (Fig. 4). During a 10-min pulse labeling with [^{35}S]methionine, 118K and 43K antigens were seen. With longer pulse labeling, the quantity of both antigens increased. During a 40-min pulse, the 64-65K antigen and the 120K antigen appeared for the first time. With even longer pulse times, all antigens increased in amount except the 43K antigen, which was present in maximum quantity at 50 min, diminished at 60 and 75 min, and totally disappeared during a 90-min pulse.

A pulse-chase experiment was conducted in an effort to determine whether the 120K antigen emerged from the 118K antigen. Again, during a 10-min pulse with [^{35}S]methionine the 118K and 43K antigens were detected. After a 3-h chase

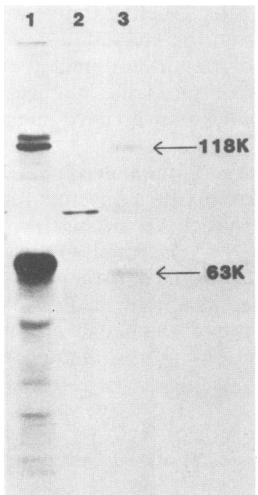

FIG. 5. Immunoprecipitation reaction of monoclonal antibody 3C7 with VZV-infected cells treated with monensin. At 48 h postinfection, cells were labeled with [^{35}S]methionine for 20 h in the presence of monensin. Tracks: 1, untreated VZV-infected cells; 2, uninfected cells treated with monensin; 3, VZV-infected cells treated with monensin.

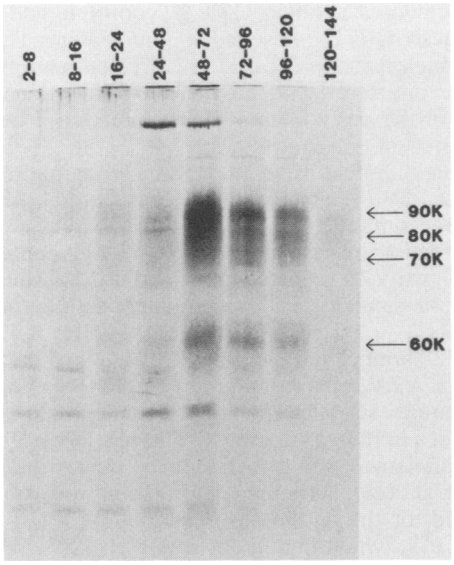

FIG. 6. Immunoprecipitation reactions of monoclonal antibody 3G8 with VZV-infected cells labeled with [^{35}S]methionine at the time intervals (hours) postinfection indicated above each lane.

the 120K and 64-65K antigens were seen, and a 24-h chase did not reveal any new polypeptides.

To further differentiate between precursor and mature glycoproteins, we treated infected cells with the glycosylation inhibitor monensin and labeled them with [^{35}S]methionine for 20 h. Monensin acts at the Golgi apparatus, inhibiting any Golgi-dependent glycosylation from occurring. Monoclonal antibody 3C7 immunoprecipitated the 118K antigen and a 63K antigen, but not the 120K or 64-65K antigen, from monensin-treated infected-cell extracts (Fig. 5). These results suggest that the 120K antigen is derived by glycosylation of the 118K antigen and that the 63K antigen may be a precursor of the 64-65K antigen. The 64-65K antigen was not an artifact resulting from cleavage of higher-MW antigens by 2-mercaptoethanol in the disrupting buffer, as it was also present in the absence of 2-mercaptoethanol (data not shown).

Group 3 monoclonal antibodies 3G8 and 4E6. Antibodies 3G8 and 4E6 both neutralized VZV only in the presence of complement. The isotype of 3G8 was IgG2b, and that of 4E6 was IgG1. When these antibodies were examined in immunoprecipitation reactions with [^{35}S]methionine-labeled infected cells, at least five broad polypeptide bands centering at 90K, 80K, 70K, 66-67K, and 60K were precipitated. With [^{14}C]glucosamine-labeled infected cells, six broad glycoprotein bands centering at 90K, 80K, 70K, 66-67K, 60K, and 50K were immunoprecipitated (Fig. 2).

The kinetics of synthesis of the polypeptides that reacted with the 3G8 antibodies was studied as described above for the group 2 antibodies. Three polypeptides with MWs of 90,000, 70,000, and 60,000 were seen as early as 2 to 8 h postinfection; the concentrations of these antigens reached maximum levels at 48 to 72 h postinfection (Fig. 6).

To determine how the antigens were related to one another in their processing and synthesis, infected cells were pulse labeled with [^{35}S]methionine for 10 to 90 min, and the cell lysates were immunoprecipitated with 3G8 antibodies and analyzed by SDS-PAGE (Fig. 7). During a 10-min pulse, a heavy precipitate was seen at 70K and four lighter bands were seen at 67K, 66K, 50K, and 40-43K. During a 20-min pulse, the level of all of these antigens had increased. During a 30-min pulse, an additional band could be identified at 90K, and during a 40-min pulse, a new 65K antigen was evident. During 50- to 75-min pulses, nine bands were precipitated at 90K, 80K, 70K, 69K, 68K, 66K, 60K, 50K, and 45-43K. During a 90-min pulse, the 69K and 40-43K bands had disappeared. Similar results were obtained when the pulse time was increased to 3 h.

Figure 8 shows the results of pulse-chase studies. When the polypeptides were chased for 3 h, four bands at 90K, 80K, 70K, and 60K were identified, and after a 24-h chase only three bands, at 90K, 80K, and 60K, were identified. The 70K antigen which was identified initially had completely disappeared after a 24-h chase.

Effect of inhibitors of glycosylation on antigens precipitated by monoclonal antibodies. Studies were performed to determine whether differing levels of glycosylation of the viral polypeptides might result in antigens of differing electrophoretic mobilities or differing antigenic specificities, which would appear as multiple bands in immunoprecipitation and SDS-PAGE analysis with the 3G8 monoclonal antibodies. Infected-cell cultures were treated with the glycosylation inhibitor 2-deoxyglucose, monensin, or tunicamycin and labeled with [^{35}S]methionine as described above.

Cell lysates from treated cultures were reacted with 3G8 hybridoma cell culture fluids or with mouse immune ascitic fluids, and the precipitates were subjected to SDS-PAGE (Fig. 9). Major changes occurred in the electrophoretic mobilities of the glycopeptides in cultures treated with inhibitors. In untreated VZV-infected cells, the major polypeptide precipitated had an MW of 90,000, whereas in all inhibitor-treated cultures the polypeptides had lower MWs.

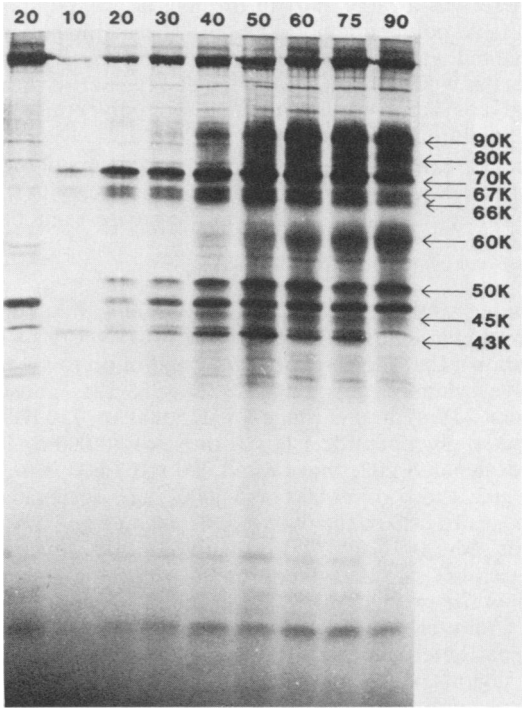

FIG. 7. Immunoprecipitation reactions of monoclonal antibody 3G8 with VZV-infected cells pulse labeled with [^{35}S]methionine for 10 to 90 min. At 48 h postinfection, the cells were pulse labeled for the times (in minutes) indicated above each lane. The first lane contained an uninfected control.

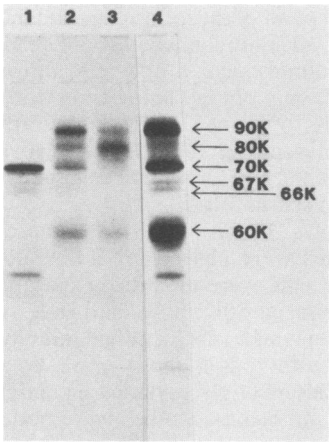

FIG. 8. Pulse-chase labeling of polypeptides in VZV-infected cells immunoprecipitated with monoclonal antibody 3G8. Cells were labeled with [^{35}S]methionine at 48 h postinfection. Tracks: 1, 10-min pulse; 2, 3-h chase; 3, 24-h chase; 4, 3-h pulse.

In cultures treated with monensin, five polypeptides were identified, at 80K, 70K, 68K, 58K, and 55K; most of the precipitated protein was at 70K. As it was previously shown that the 70K glycopeptide appeared first during pulse labeling and chased to higher MWs, it would appear that monensin treatment inhibited glycosylation, and the polypeptide remained at 70K. Also, synthesis of the 60K glycopeptides was inhibited, and polypeptides with lower MWs, the 58K and 55K polypeptides, appeared; these may be unglycosylated forms of the 60K polypeptide.

From cells treated with 2-deoxyglucose, we precipitated 90K, 85K, 80K, 70K, 65K, 58K, 57K, and 40K polypeptides, and there was a large shift in the migration and spread of precipitable polypeptides in the gel. The major polypeptide precipitated was the 80K polypeptide, and there were trace amounts at 90K and 40K. Although deoxyglucose prevented most glycosylation to produce the 90K polypeptide, it was not as inhibitory as monensin was.

In cells treated with tunicamycin, only four polypeptides were precipitated, at 85K, 80K, 68K, and 40K, with the 80K and 68K peptides being present in the greatest amounts.

DISCUSSION

Previous studies that used SDS-PAGE and immunoprecipitation to characterize the glycoproteins of the VZV virion have shown that the glycoproteins band in up to six regions in polyacrylamide gels (10, 12, 24, 25, 29). Those with apparent MWs in the range of 115,000 to 120,000 were designated glycopeptide 1 (gp1), those of 80,000 to 100,000 were designated gp2, those of 64,000 to 67,000 were designated gp3, those of 59,000 or 63,000 were designated gp4, and those of 52,000 or 55,000 were designated gp5 (24, 25). In addition, Shiraki et al. (25) designated a 45K glycopeptide gp6. Analysis of VZV glycoproteins with monoclonal antibodies in the present study and in those of Grose et al. (11) and Okuno et al. (18) has indicated the antigenic and biological heterogeneity of glycoproteins banding in these major regions.

The glycoproteins included in gp1 appear to contain some determinants that are important sites for viral neutralization and others that are not. The characteristics of our group 1 monoclonal antibodies 4B7 and 8G9 were remarkably similar to those of a clone recently described by Grose et al. (11). They precipitated a single 118K glycoprotein and had neutralizing activity in the absence of complement. The epitope against which these antibodies are directed must be located within, or interfere with, an important functional domain involved in the early events of the infection process, i.e., viral adsorption or penetration.

An interesting feature of the 118K glycoprotein immunoprecipitated by the group 1 monoclonal antibodies was its rapid disappearance from infected cells. Shiraki and Takahashi (26) have reported that 115K and 45K glycoproteins are excreted from VZV-infected cells, and it is possible that the former is the same as the 118K polypeptide identified in our studies.

Group 2 monoclonal antibody 3C7 lacked neutralizing activity for VZV, and it immunoprecipitated glycoproteins with apparent MWs in the gp1 complex and also 65K polypeptides in the gp3 complex. The reactivity of antibody 3C7 was similar to that of some of the clones described by Okuno et al. (18), although neutralizing activity was not determined for the antibodies described by them. Group 2 antibody 3C7, which immunoprecipitated 120K, 118K, and 65K polypeptides, was similar to clone 8 of Okuno et al., which precipitated 116K, 106K, and 64K polypeptides. In pulse-chase experiments, the first detectable antigens precipitated by the 3C7 antibody were a major 118K antigen and a minor 43K antigen. The 64K antigen next appeared, whereas the 43K antigen diminished and then disappeared, and finally the 120K antigen appeared. Similarly, Okuno et al. reported that in pulse-chase experiments with their clone 8 antibody, the first antigen was a 106K antigen, followed by the 64K antigen and finally the 116K antigen. If our 3C7 antibody and the clone 8 antibody are in fact identical in their specificities, it may be that the differences in MW of the larger polypeptides precipitated are attributable to minor differences in the conditions used for SDS-PAGE or in the host cells used for virus propagation. Okuno et al. showed

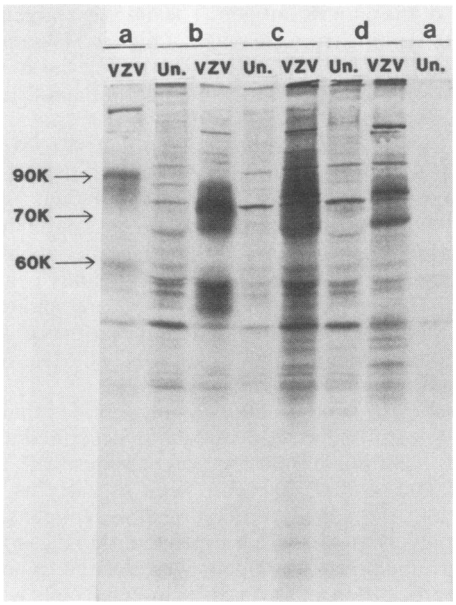

FIG. 9. Immunoprecipitation reactions of monoclonal antibody 3G8 with VZV-infected and uninfected (Un.) cells labeled at 48 h postinfection with [^{35}S]methionine for 20 h with or without a glycosylation inhibitor. Lanes: a, untreated; b, monensin; c, 2-deoxyglucose; d, tunicamycin.

that the 64K polypeptide, but not the 116K or 106K polypeptide, was expressed on the surface membrane of VZV-infected cells, and they postulated that the 64K antigen may be a cleavage product of a larger antigen which is expressed on the cell surface. Tryptic peptide analysis can be expected to indicate whether the antigens are derived from the same precursor. Although similar in MW, the 118K antigen precipitated by the group 2 3C7 antibody differed from the 118K antigen precipitated by the group 1 4B7 and 8G9 antibodies in biological activity as well as in antigenic specificity. The 118K antigen recognized by antibody 3C7 did not contain sites critical for virus neutralization, and it differed further from the 118K antigen recognized by group 1 antibodies in that it appeared to be processed into a 120K antigen. The 118K antigen which contains a critical site for neutralization might be designated 118K-N to distinguish it from the 118K antigen recognized by the nonneutralizing 3C7 antibody.

Grose et al. (11) identified monoclonal antibodies without neutralizing activity for VZV which coprecipitated 98K glycopeptides, which would fall into the gp2 complex, and 62K glycopeptides, which would fall into the gp4 range. These glycoproteins were shown to be prominent constituents of the infected-cell membrane (27).

Group 3 monoclonal antibodies 3G8 and 4E6 were able to neutralize VZV only in the presence of complement and immunoprecipitated several polypeptides ranging in MW from 90,000 to 40,000. Although both monoclonal antibodies reacted with the same antigens, at similar immunoglobulin concentrations the immunoprecipitation reactivity of the 3G8 clone was much stronger than that of the 4E6 clone (c.f. Fig. 2). Furthermore, immunofluorescence staining reactions with 4E6 antibodies were less intense than those with 3G8 antibodies, even with an excess of 4E6 antibody. One possible explanation for this could be that the two antibodies are directed against different epitopes on the same glycopeptide, and the epitope against which 3G8 antibodies are directed is more accessible under the mildly denaturing conditions of the immunoprecipitation reaction. Another possibility is that the affinity of the two antibodies is different, particularly since they belong to different subclasses of mouse IgG.

In kinetics studies with the 3G8 antibody, a 70K antigen was the first to appear, together with several minor polypeptides with lower MWs. The latter disappeared over the course of infection, with an accumulation of antigens of higher MWs, 80,000 and 90,000. In pulse-chase experiments, the early-appearing 70K antigen disappeared, and after a 24-h chase only 90K, 80K, and 60K antigens were evident. The results of experiments with inhibitors of glycosylation would suggest that the early-appearing 70K antigen which chased into 80K and 90K polypeptides may have been a precursor of the latter and also that the polypeptides with MWs below 60,000 may have been precursors of the 60K antigen. The lower-MW antigens were not a result of cleavage of higher-MW antigens by 2-mercaptoethanol in the disrupting buffer, since they were also present in the absence of 2-mercaptoethanol (data not shown). Monoclonal antibodies that reacted with a similar range of VZV polypeptides were reported by Okuno et al. (18), and the results of their pulse-chase experiments were somewhat similar to ours in that an early 75K antigen chased into 94K and 83K polypeptides and a 49K polypeptide chased into a 55K band. The biological activity of their monoclonal antibodies was not reported.

It is interesting that monoclonal antibodies with complement-independent neutralizing activity reacted with a single 118K polypeptide, whereas those with neutralizing activity that was totally complement dependent reacted with polypeptides ranging in MW from 90,000 to 50,000, apparently at varying levels of glycosylation. These antibodies will be useful in studies aimed at determining the mechanisms by which these two types of neutralization occur.

The three inhibitors of glycosylation used in the present study have different mechanisms of action. The sugar analog 2-deoxyglucose reduces glycosylation of viral proteins through incorporation into the glycoproteins in place of mannose; it thus prevents addition of the complete mannose core and oligosaccharide chain elongation (4, 9). Tunicamycin is a glucosamine-containing antibiotic that inhibits the transfer of *N*-acetylglucosamine-1-phosphate from UDP-*N*-acetylglucosamine to dolichol monophosphate and thereby blocks the formation of protein-carbohydrate linkages of the *N*-glycosidic type (16, 17). Monensin is a monocarboxylic ionophore which appears to disrupt ionic gradients across intracellular and plasma membranes; such gradients are important in the maturation and transport of viral glycoproteins to the cell surface, as well as in virus-induced cell fusion (13, 14). Our results with the three inhibitors of glycosylation would suggest that glycosylation of VZV is similar to that of herpes simplex virus in several respects. First, the inhibitors did not destroy the antigenicity of the glycoproteins immunoprecipitated by monoclonal antibodies 3C7 and 3G8, but only lowered their apparent MWs. This would suggest that the antigenic domains against which the monoclonal antibodies are directed are located within the polypeptide backbone of the glycoproteins and that Golgi-dependent glycosylation does not alter the antigenic conformation of the major VZV antigens (9, 17). Glycosylation of herpes simplex virus glycoproteins is thought to occur via both *N*-acetylglycosamine and *O*-acetylglycosamine (19, 20). 2-Deoxyglucose, which substitutes for mannose, did not completely inhibit the glycosylation of VZV glycoproteins; this may be because the mannose core was not completely removed, resulting in partially glycosylated intermediates (4, 9). Since tunicamycin inhibited the maturation of some VZV glycoproteins but also shifted the MWs of others, it would appear that VZV glycosylation occurs via both *N*-acetyl and *O*-acetyl glycosidic linkages, as previously reported for herpes simplex virus (19, 20).

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