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Effect of Tunicamycin on the Synthesis of Herpes Simplex Virus Type 1 Glycoproteins and Their Expression on the Cell Surface

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Herpes simplex virus specifies five glycoproteins which have been found on the surface of both the intact, infected cells and the virion envelope. In the presence of the drug tunicamycin, glycosylation of the herpes simplex virus type 1 glycoproteins is inhibited. We present in this report evidence that the immunological specificity of the glycoproteins designated gA, gB, and gD resides mainly in the underglycosylated "core" proteins, as demonstrated by the immunoblotting technique. We showed also that tunicamycin prevented exposure of the viral glycoproteins on the cell surface, as the individual glycoproteins lost their ability to participate as targets for the specific antibodies applied in the antibody-dependent, cell-mediated cytotoxicity test. Immunocytolysis was reduced between 73 and 97%, depending on the specificity of the antibodies used. The intracellular processing of the herpes simplex virus type 1-specific glycoprotein designated gC differed from the processing of gA, gB, and gD, as evidenced by the identification of an underglycosylated but immunochemically modified form of gC on the surface of infected cells grown in the presence of tunicamycin.

In the course of productive infection of cells in culture, herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) specify several glycosylated proteins, designated gA, gB, gC, gD, and gE, which become incorporated into cellular membranes and into virion envelopes (1, 6, 21, 22, 25, 29). In the infected host, the glycoproteins induce the production of neutralizing, precipitating, and cytolytic antibodies (11, 14, 29). gC, gD, and gE each carry a unique set of antigenic determinant sites (3, 11), whereas gA and gB appear to be immunologically related, as demonstrated recently by the use of monoclonal antibodies (18). The glycoproteins gA, gB, gD, and gE of HSV-1 and HSV-2 specify antigen determinant sites which are common to both HSV-1 and HSV-2 proteins (4, 13, 18), but the glycoproteins also carry antigen determinant sites which are unique for either HSV-1 or HSV-2 (4, 18). The antigen determinant sites of HSV-1 gC are type specific (28), and HSV-2 gC also specifies antigen determinant sites specific for HSV-2 (S. Bacchetti, personal communication).

The glycosylation of the viral proteins is a stepwise process by which oligosaccharide chains are added to the proteins in discrete steps (3, 6, 26). The fully glycosylated proteins gA, gB, gC, gD, and gE of HSV-1 and HSV-2, located in the plasma membrane and in the virion envelope, were identified by various immunological methods (11, 21, 26). The manner in which the precursors are processed into the fully glycosylated product is not known in detail, but glycosylation of gA, gB, gC, gD, and gE apparently involves a dolichol-oligosaccharide intermediate, and the carbohydrate chains are therefore bound through *N*-glycosidic bonds to asparagine (5, 19). It has recently been reported that HSV-1 gC may have carbohydrate chains linked through *O*-glycosidic bonds to serine or threonine (5, 16). The glycosylation of all of the viral proteins can, however, be inhibited by tunicamycin (TM), which inhibits the dolichol-dependent transfer of the mannose-rich backbone of the carbohydrate chains to the proteins (5).

In the present study, the immunoblotting technique was used for the analysis of the immunochemical reactivity and specificity of the HSV-1 unglycosylated proteins which accumulated in the presence of TM (15, 19). Data are also presented which support the conclusion that glycosylation of the HSV-1 proteins is necessary mainly for their intracellular transport to and expression on the surface of infected cells.

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MATERIALS AND METHODS

Tissue culture cells. Monolayers of Vero cells were grown and maintained in minimal essential medium

(MEM) with 10% heat-inactivated fetal calf serum (FCS).

Infection of cells. Cell monolayers in 25-cm^2 tissue culture flasks were infected at a multiplicity of infection (MOI) of either 1 or 10 PFU of HSV-1 strain F or (HFEM)tsB5 per cell. HSV-1 (HFEM)tsB5 was a gift from A. Buchan, Department of Microbiology, University of Birmingham, England. The mutant strain was defective in the synthesis of glycoprotein gB at 39°C (8). The virus was allowed to adsorb for 1 h at 37°C in 1.0 ml of MEM with 1% FCS. At the end of the adsorption period, the inoculum was replaced by 5 ml of MEM with 1% FCS. Where specified in the text, the overlayer medium was supplemented with 3 μg of TM per ml, which gave the optimal inhibition. TM was a generous gift from Eli Lilly & Co., Indianapolis, Ind.

Preparation of virus proteins for SDS-polyacrylamide gel electrophoresis. At 18 h postinfection, cells infected at an MOI of 10 PFU per cell were washed once in phosphate-buffered saline after the medium had been aspirated. The cells were dissolved in 0.5 ml of a disruption mixture with 2% (wt/vol) sodium dodecyl sulfate (SDS) and 5% (vol/vol) 2-mercaptoethanol (11). For radiolabeling of the proteins with ^{14}C-amino acids, infected cells were overlayered with 2.5 ml of MEM with 1/10 the normal concentration of the three amino acids isoleucine, leucine, and valine and supplemented with 2 μCi of each of the three ^{14}C-labeled amino acids (specific activities: isoleucine, 345 mCi/mmol; leucine, 339 mCi/mmol; valine, 285 mCi/mmol; Amersham Corp., England) per ml of medium. The infected cells were labeled in 2-h pulses from 6 to 8, 10 to 12, or 14 to 16 h postinfection. Two cultures were labeled in parallel: one was grown under the usual conditions, but the other was grown and labeled in medium with TM. Labeling of HSV-1-infected cultures with D-[^{14}C]glucosamine was done by the addition of 1 μCi of D-[^{14}C]glucosamine per ml of medium (specific activity, 756.86 Ci/mmol; Amersham) in 2.5 ml of maintenance medium from 6 to 18 h postinfection. The viral proteins were numbered by the nomenclature system of Morse et al. (10), and the glycoproteins were numbered by the nomenclature system adopted at the Herpesvirus Workshop held at the Cold Spring Harbor Laboratory in 1979.

SDS-polyacrylamide gel electrophoresis. HSV-infected cell extracts were separated in 9.25% (wt/vol) acrylamide cross-linked with 0.24% (wt/vol) *N,N*-diallyltartardiamide (Bio-Rad Laboratories, Richmond, Calif.). Buffer conditions and processing of the gels for autoradiography were as described by Morse et al. (10).

Immunoblotting. The viral proteins were separated in gels prepared as described above but supplemented with 1% (wt/vol) agarose (Litex, Copenhagen, Denmark). The processing of the gels for transfer is described in detail elsewhere (15, 20). The elution of the viral proteins and their binding to diazobenzyloxymethyl paper were done as described by Norrild et al. (15).

Paper strips with immobilized HSV-1 protein were incubated with hyperimmune rabbit sera made either to all of the antigenic HSV-1 proteins or to individual glycoproteins produced as described below. The sera were diluted 1/10 in a 0.05 M Tris-hydrochloride buffer (pH 7.4) containing 0.15 M NaCl, 0.005 M EDTA, 0.25% (wt/vol) gelatin, and 0.05% (vol/vol) Triton X-100. The binding was carried out for 5 to 6 h by incubation at 37°C. The strips were washed for 18 h and incubated for 2 h with 0.25 μCi of ^{125}I-labeled protein A (specific activity, 30 mCi/mg of protein; Amersham). After a final wash in buffer as described above but supplemented with 1 M NaCl and 0.4% (wt/vol) Sarkosyl, the strips were dried.

Autoradiography was done on Kodak XPR-1 film exposed for 3 to 7 days at 4°C.

ADCC test. (i) Target cells. HSV-1-infected cells (MOI, 1 PFU per cell) grown either in MEM or in MEM with 3 μg of TM per ml were trypsinized at 18 h postinfection in 0.25% (wt/vol) trypsin. The cells grown without TM were washed twice in MEM with 1% FCS and labeled with 100 μCi of ^{51}Cr (specific activity, 0.487 mCi/μg; Amersham) in 1.0 ml of MEM. After 1 h at 37°C, the labeled cells were washed through a 4-ml cushion of heat-inactivated FCS. The cell pellet was suspended in MEM with 10% heat-inactivated FCS to a final concentration of 5×10^4 cells per ml (14, 24). Cells grown in TM-containing medium were prepared as described above, but all media were supplemented with 3 μg of TM per ml.

(ii) Effector cells. Human mononuclear cells were prepared from peripheral blood from donors at the Rigshospitalet, Copenhagen. Buffy coat cells were stored at 4°C for 24 h at the most before use, and Ficoll-Hypaque gradients were made with 10 ml of Ficoll-Hypaque overlayered with 30 ml of the heparinized blood sample diluted 1:1.5 in 0.9% (wt/vol) NaCl (24). The interphase cells were washed three times in Hanks balanced salt solution and finally suspended in MEM with 10% heat-inactivated FCS to a concentration of 2.5×10^6 cells per ml.

(iii) Antisera. Hyperimmune rabbit sera containing antibodies against HSV-1 proteins were prepared as described in detail elsewhere (29, 30).

Serum 0 was preimmune rabbit serum.

Serum 1 was a polyspecific serum made by infecting rabbits with HSV-1.

Sera 11, 8, and 6 were all hyperimmune sera reactive to individual glycoproteins (29). Serum 11 reacted to the mixture of gA and gB (antigen 11; Ag-11), 8 reacted to gD (Ag-8), and 6 reacted to gC (Ag-6). The terminology for the antigens in parentheses is based on the identification of the viral glycoproteins by crossed immunoelectrophoretic analysis (27).

The antibody-dependent, cell-mediated cytotoxicity (ADCC) test was made in a 1.0-ml reaction volume: 0.4 ml of target cells (2×10^4), 0.4 ml of effector cells (10^6), and 0.2 ml of rabbit serum in a final dilution of 1:100 in the reaction mixture. TM (3 μg/ml) was included in the reaction mixture during incubation for 6 h at 37°C. After the mixture was centrifuged, 0.5 ml of the supernatant was harvested. Supernatant (0.5 ml) and 0.5 ml of the corresponding pellet from each sample were counted in a Beckman gamma counter, and the percentage of ^{51}Cr release was calculated for samples made in triplicate. The formula used for the calculations was: $[(2 \times \text{cpm(S)})/(\text{cpm(S)} + \text{cpm(P)})] \times 100$, where S is the supernatant and P is the pellet. The percentage of specific release was the difference between the percentage of release in the presence of sera positive for antibodies to HSV-1 minus the percentage of release with preimmune serum in the test.

^{125}I iodination of the surface of intact infected cells. Cells in monolayer culture, infected at an MOI of 10

PFU per cell and maintained either in MEM with 1% FCS or in MEM with 1% FCS and 3 μg of TM per ml, were washed extensively in phosphate-buffered saline at 18 h postinfection. The labeling of the cell surface was done as detailed elsewhere and by the method of Morrison (9, 12). After being extensively washed in phosphate-buffered saline, the labeled, intact cells were solubilized in a 0.020 M glycine–0.0076 M Tris buffer (pH 8.6) with 1% (vol/vol) nonionic detergent Triton X-100. The cell extract was sonicated and centrifuged at 100,000 × *g* for 1 h at 4°C in an SW60 rotor.

Crossed immunoelectrophoretic analysis of soluble HSV-1 proteins. We used the modified method described by Norrild et al. (12) for the crossed immunoelectrophoretic analysis. Briefly, the HSV-1 soluble proteins were electrophoretically separated in a 1% (wt/wt) agarose gel (the one-dimensional gel). The separated proteins were immunoprecipitated by electrophoresis into an agarose gel supplemented with serum 1, which contained antibodies to all of the HSV-1 proteins (the two-dimensional gel). In the present study, an intermediate gel was, when specified in the text, inserted between the one- and the two-dimensional gels. The intermediate gel was supplemented with serum 6. The quantity of antigen and antibodies applied to the gel is indicated in the figure legends.

RESULTS

HSV-1 proteins synthesized during inhibition with TM. TM has been reported as an efficient inhibitor of the glycosylation of HSV-1 proteins in infected baby hamster kidney cells and in HEp-2 cells (19; R. J. Courtney, personal communication). In the present work, the synthesis and immunochemical specificity of unglycosylated viral proteins was studied. HSV-1-infected Vero cells were labeled with ^{14}C-labeled isoleucine, leucine, and valine in 2-h pulses, from 6 to 8, 10 to 12, or 14 to 16 h postinfection, with and without TM added to the maintenance medium immediately after the adsorption period. Mock-infected cells were labeled from 6 to 8 h postinfection as a control. As was found in work done at other laboratories, most of the virus-specified proteins made in infected cells (ICPs) showed the same electrophoretic mobility independent of the presence of TM in the medium (19). Our data confirm and extend these findings, showing that ICPs 10 (gB), 11 (gA), 12, 14, 15, 29 (gD), and 32/33 disappeared in cultures grown with TM but that polypeptides with a higher electrophoretic mobility were concurrently identified (Fig. 1). The apparent molecular weights of the most intensively labeled proteins which accumulated during growth with TM were 102,000 (102K), 101K, 98K, 82K, 48K, and 44.5K, respectively, but polypeptides with molecular weights of 73.5K, 60K, and 59K were found as well (Fig. 1d, e, f, and g). All of the glycoproteins were synthesized at 6 h postinfection, but the intensity of the label increased when the pulse-labeling was done later in the infectious cycle (Fig. 1b, c, d, and e).

Immune reactivity of the immobilized HSV-1 polypeptides made with and without TM inhibition. The antigen relationship between ICPs 10 and 11 (gB and gA) and the unglycosylated polypeptides with molecular weights of 102K, 101K, and 98K, and between ICP 29 (gD) and the polypeptides with molecular weights of 48K to 44.5K, was demonstrated by the immune reactivity of the immobilized polypeptides of HSV-1 strain F grown with and without TM. The ability of the transferred polypeptides to bind the antibodies present in hyperimmune rabbit serum made against HSV-1 proteins (serum 1) allowed the detection of approximately 15 of the viral ICPs; but ICPs 10, 11, and 29 could be detected only in extracts from cells grown without TM (Fig. 2a). ICPs 10 and 11 and ICP 29 were apparently translocated in TM-treated cells to the positions corresponding to ICPs with molecular weights in the range of 102K to 98K and 48K to 44.5K, respectively (Fig. 2b).

The antigenic identity between ICPs 10 and 11 (gB and gA) and the polypeptides with molecular weights of 102K to 98K and between ICP 29 (gD) and the polypeptides with molecular weights of 48K to 44.5K was confirmed by the binding of specific antibodies to immobilized HSV-1 proteins. Antibodies reactive to glycoproteins gA, gB, and gD (a mixture of sera 11 and 8) or to gA and gB only (serum 11) were bound to the paper strips (Fig. 2c, d, e, and f).

The immune reactivity of HSV-1 proteins made during inhibition of the glycosylation by TM showed that gA and gB were translocated to the position corresponding to the ICPs with molecular weights of 102K to 98K (Fig. 2d and f), whereas proteins with molecular weights of 48K to 44.5K bound antibodies to gD (Fig. 2c and d). Because of the limited resolution of the SDS-polyacrylamide-agarose gels, only one protein band which bound antibodies against the mixture of gA and gB and one band which bound antibodies reactive to gD were identified after the immune reaction.

It should be noted that synthesis of both gA and gB was inhibited in the presence of TM and that the unglycosylated precursor molecules were identified as one band in the present test, although three polypeptides with molecular weights of 102K, 101K, and 98K, respectively, were identified in SDS-polyacrylamide gels (Fig. 1g). Either the polypeptide cores of gA and gB are very similar in size or gA and gB may be processed from a common core protein (98K) along different routes of glycosylation.

The glycoprotein gC of HSV-1 and its underglycosylated precursor could not be demonstrat-

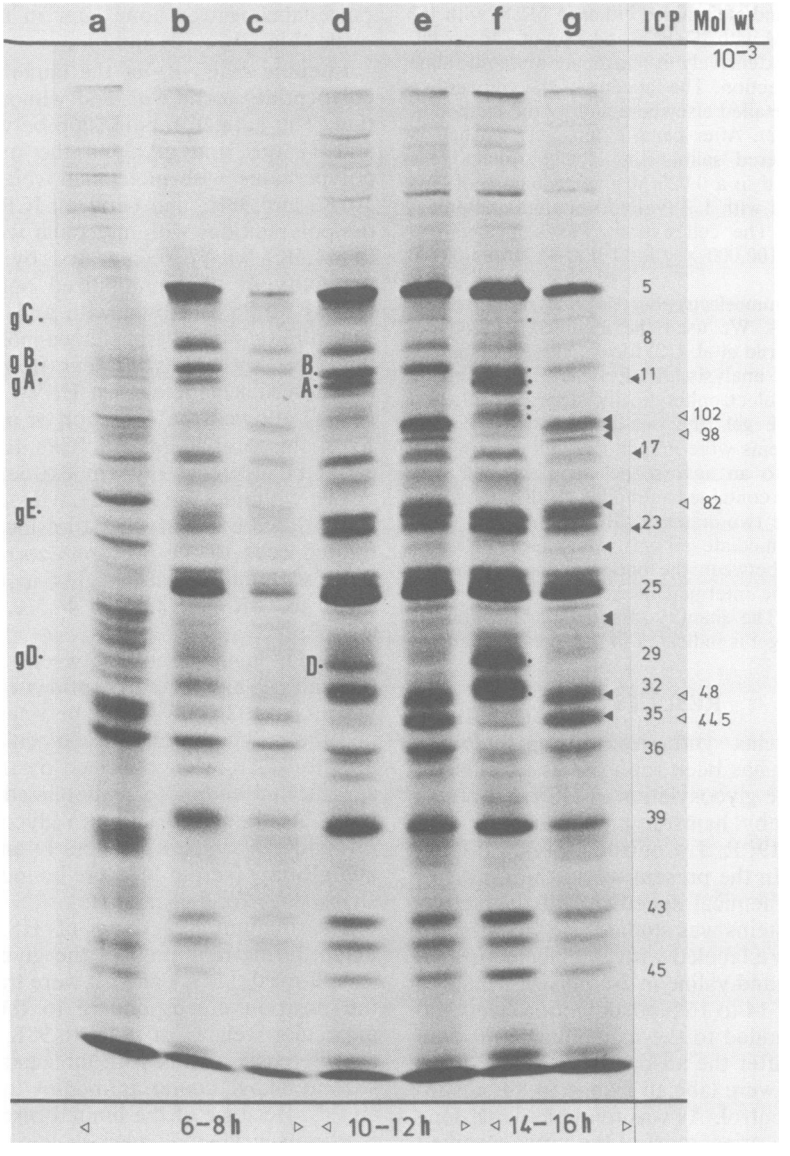

FIG. 1. Autoradiographic image of HSV-1 polypeptides separated in an SDS-polyacrylamide gel. The HSV-1-infected cells were grown either with or without TM and pulse-labeled with ^{14}C-amino acids at the times indicated. Lanes: (a) proteins extracted from mock-infected cells labeled from 6 to 8 h postinfection; (b, d, and f) proteins extracted from cells grown in the absence of TM and labeled from 6 to 8, 10 to 12, or 14 to 16 h postinfection; (c, e, and g) proteins extracted from cells grown in the presence of 3 μg of TM per ml of medium and labeled from 6 to 8, 10 to 12, or 14 to 16 h postinfection. The proteins which disappear during growth in medium with TM are indicated by dots in lane f. The translocated underglycosylated proteins which accumulated in the presence of TM are indicated by arrows in lane g.

ed in the immobilization test, because none of the antibody preparations reactive to gC in immune precipitation (serum 6) reacted in the immunoblotting test (15). That gC did bind to the paper was shown by transfer of [^{14}C]glucosamine-labeled gC (unpublished data).

Inhibition by TM of expression on the cell surface of the antigen determinants specified by gA, gB, gC, and gD. HSV-1-infected cells in which gA and gB (antigen Ag-11), gC (antigen Ag-6), and gD (antigen Ag-8) are incorporated into the plasma membrane have previously been shown to participate as target cells in antibody-dependent, cell-mediated cytolysis (14). Any

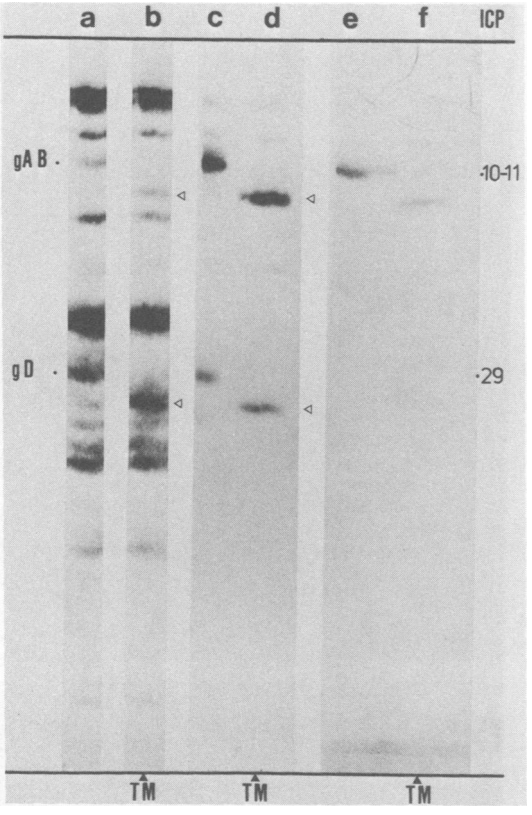

FIG. 2. The autoradiographic image of HSV-1 proteins immobilized onto diazobenzyloxymethyl paper and made to react with hyperimmune rabbit serum followed by binding of ^{125}I-labeled protein A. Lanes a, c, and e: HSV-1 proteins extracted from cells grown without TM are reacted with serum 1, sera 11 and 8, or serum 11, respectively. Lanes b, d, and f: HSV-1 proteins extracted from cells grown with TM are reacted with serum 1, sera 11 and 8, or serum 11, respectively. Note the translocation of glycoproteins gA, gB, and gD in the presence of TM, as indicated by the arrows in lanes b and d.

one of the glycoprotein antigen structures is capable of functioning as a target for immune cytolysis, as was shown when antibodies reactive to only one of the antigens were applied separately in the test. The requirement that the proteins be fully glycosylated for transport to the surface of the cell was studied by incubation of the HSV-1-infected cells with TM during the entire infectious cycle. When TM-inhibited, HSV-1-infected cells were used as the target cells in the ADCC test with serum 1, 11, 8, or 6, the specific ^{51}Cr release ranged from 1.1 to 14.2% (Table 1). When uninhibited HSV-1-infected cells were used as the target cells, the specific ^{51}Cr release varied between 39.3 and 52.3% (Table 1).

TM thus inhibited the surface expression of the antigen-reactive glycoprotein domains from 73 to 97%. Although TM was added to the maintenance medium during the ADCC reaction in a quantity sufficient to inhibit glycosylation, a small number of infected cells were still lysed in the presence of serum 1 or 8. The efficiency of inhibition by TM was tested in each experiment, and no incorporation of [^{14}C]glucosamine in a cell culture grown in parallel to the culture used for the ADCC test could be demonstrated when the proteins were analyzed on SDS-polyacrylamide gels (data not shown).

Because of the residual immune cytolysis measured when TM-inhibited cells were incubated in the presence of sera 1 and 8 in the ADCC test, the surface location of the viral proteins was further analyzed by iodination of the surface of intact infected cells with ^{125}I. The viral glycoprotein antigens designated Ag-11 (gA and gB), Ag-8 (gD), and Ag-6 (gC) were all found on the surface of cells grown without TM (Fig. 3A). In cells inhibited by TM, a protein with immunological cross-reactivity to Ag-6 (gC) of HSV-1 was available for labeling on the surface of the cells. This protein was precipitable with serum 6 (reactive to gC) when cellular extracts were analyzed in the crossed immunoelectrophoretic test with serum 6 in the intermediate gel (Fig. 3C and D). Besides Ag-6, only trace amounts of label were found in the proteins present in the antigen structure Ag-8 (Fig. 3C). This antigen was identified by the use of specific antibodies in the intermediate gels (data not shown).

TABLE 1. ADCC measured by HSV-infected target cells grown with and without TM

Antiserum	% ^{51}Cr release[a]		% Reduction
	Without TM	With TM	
None	12.6 ± 4.9	29.9 ± 6.6	
Preimmune (0)	16.7 ± 1.2	27.9 ± 0.7	
1	63.9 ± 0.9 (47.3 ± 1.1)	40.6 ± 2.6 (12.7 ± 1.9)	73.2
11	61.4 ± 2.6 (44.8 ± 2.0)	32.4 ± 0.9 (4.5 ± 0.8)	90.1
8	68.9 ± 2.3 (52.2 ± 1.9)	42.1 ± 2.6 (14.2 ± 1.9)	72.9
6	56.0 ± 5.7 (39.3 ± 4.1)	29.0 ± 3.4 (1.1 ± 2.4)	97.2

[a] Mean of three observations ± standard deviation. The specific ^{51}Cr release is shown in parentheses.

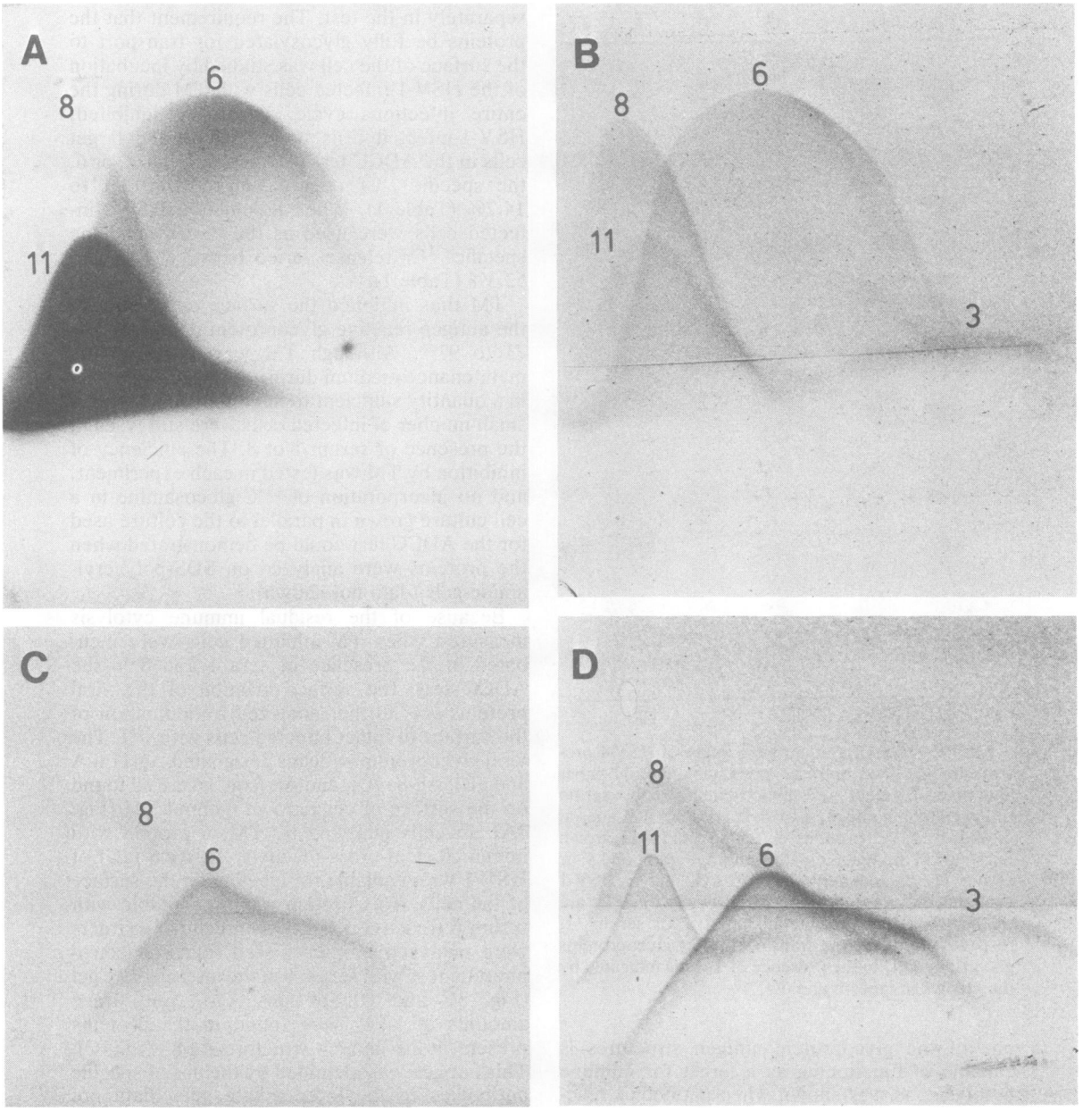

FIG. 3. Crossed immunoelectrophoretic analysis of HSV-1 proteins extracted from infected cells. The surface-exposed viral proteins were labeled with ^{125}I by labeling the intact infected cells grown with or without TM. Cellular extract (30 μl) was separated in the one-dimensional gel. The two-dimensional gel was supplemented with 15 μl of serum 1 per cm^2. (A) Autoradiographic image of the immunoprecipitates formed with cellular extracts made from cells grown in the absence of TM. The intermediate gel contained serum 0 (15 μl/cm^2). (B) The same plate as shown in A, but stained with Coomassie brilliant blue. (C) Autoradiographic image of the immunoprecipitates formed with cellular extracts made from cells grown in the presence of TM. The intermediate gel was supplemented with serum 6 (15 μl/cm^2). (D) The same plate as shown in C, but stained with Coomassie brilliant blue.

The quantity of unglycosylated proteins present in the viral antigens with an immunological reactivity identical to that of gA and gB (Ag-11), gD (Ag-8), and gC (Ag-6) was similar to the quantity of gA and gB (Ag-11), gD (Ag-8), and gC (Ag-6) produced in cells not inhibited by TM;

this was demonstrated by staining the immunoprecipitates formed by crossed immunoelectrophoretic analysis of the viral proteins extracted from infected cells grown with and without TM (Fig. 3B and D).

DISCUSSION

In this paper we present data which show that unglycosylated proteins with the same immunochemical specificity as gA, gB, and gD accumulate in HSV-1-infected cells grown in the presence of TM. We show also that TM inhibited the expression of gA, gB, and gD on the cell surface, whereas gC was present in an underglycosylated form with modified immunochemical reactivity.

Immunochemical specificity of the unglycosylated viral proteins. Among the proteins which accumulated when the glycosylation process was inhibited (Fig. 1e and g), the 48K and 44.5K proteins had an immunochemical specificity identical to gD, as demonstrated by the binding of gD-reactive antibodies (serum 8) to the immobilized HSV-1 protein (Fig. 2c and d). This finding confirms data published by other laboratories (19). The reason for the appearance of two new proteins (48K and 44.5K) in the presence of TM is unclear, but the 44.5K protein most likely represents the unglycosylated precursor to gD, whereas the 48K protein was partially glycosylated (Fig. 1e and g).

Pizer et al. (19) demonstrated that an 85K protein accumulates during the growth of HSV-1-infected cells in TM and that this protein is related to gC. We identified an unglycosylated protein, 98K, which had an immunochemical reactivity similar to that of gA and gB as demonstrated by the application of specific antibodies in the immunoblotting test (Fig. 2e and f). The acrylamide-agarose gels had limited resolution; thus, we were unable to demonstrate whether, in the presence of TM, gA and gB accumulated as two different unglycosylated proteins with similar electrophoretic mobility or had a common unglycosylated form, as has been suggested by others (3).

It should be noted that, in agreement with a previous report (15), diazobenzyloxymethyl paper-bound gC and the underglycosylated protein corresponding to gC did not react in the immunoblotting test.

Transport of glycosylated viral proteins to the surface of HSV-1-infected cells. The individual glycoproteins of HSV-1 have been shown to be exposed on the surface of intact infected cells, where they are available for labeling with ^{125}I (12). The glycoproteins gA, gB, gC, and gD participate as targets for specific antibodies applied in the ADCC test (14), and our present data show that TM inhibited the surface expression of each of the glycoproteins gA, gB, and gD, only trace amounts of these proteins being available on the cell surface for iodination with ^{125}I (Fig. 3C). When TM-inhibited cells were applied in the ADCC test with specific antibodies reactive with either gA, gB, gC, or gD, no or very little immunocytolysis was demonstrable (Table 1). The residual cytolysis measured with reference serum 1 or with serum 8 might be the result of the incorporation of minute amounts of either unglycosylated or underglycosylated protein gD into the plasma membrane. This finding was consistent with the minute amount of Ag-8 seen by surface labeling (Fig. 3C). TM also inhibits the cell-mediated immunocytolysis of HSV-1-infected cells (2), which is also mediated through the participation of the viral glycoproteins as targets for the specific T-cells (7). That unglycosylated proteins with the same immunological reactivity as gA, gB, and gD could be demonstrated in extracts made from TM-inhibited, infected cells showed that the unglycosylated proteins were stable in vivo and that TM mainly inhibits the transport to and integration of the proteins into the plasma membrane. The unglycosylated proteins may have been integrated into the membrane, but the structure was thereby modified enough to prevent labeling of the proteins by iodination of the intact cells and their participation as targets for the specific antibodies applied in the ADCC test.

The protein with the immunological specificity of gC was the glycoprotein which was most abundant on the surface of the plasma membrane of the TM-treated cells, as demonstrated by ^{125}I labeling of the cell surface proteins (Fig. 3C). As the protein did not react as a target for antibodies against gC when these were applied in the ADCC test (Table 1), our finding supports the hypothesis that gC must be fully glycosylated to function as a target in the immune cytolytic test. We found that TM inhibited the incorporation of [^{14}C]glucosamine into all of the HSV glycoproteins including gC (data not shown). The major part of the carbohydrate of gC is therefore linked through *N*-glycosidic, TM-sensitive bonds; however, *O*-glycosidic-linked carbohydrate may be present. This assumption is based on the specific affinity of gC for the lectin from *Helix pommatia* (16), and, because the *O*-glycosidic bonds are not sensitive to inhibition by TM, the residual carbohydrate on gC, which is low in glucosamine content (S. Olofsson, personal communication), might be sufficient for the transport and integration of the protein into the membrane. The analysis of the linkage of the carbohydrate to the core of gC and the biological significance of the *O*-glycosidic linkage for HSV-1 need further investigation, but it should be mentioned that a glycoprotein has been identified in vaccinia virus in which 25% of the

carbohydrate was linked through *O*-glycosidic bonds and the *O*-linked sugar was a necessity for the biological function of the molecule (23).

In conclusion, our data present evidence that the antigen specificity of the HSV-1 glycoproteins gA, gB, and gD is determined mainly by the unglycosylated core proteins, but glycosylation is a necessity for the transport and integration of the viral proteins into the plasma membrane of infected cells.

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