Monoclonal Antibodies Reactive with the Surface and Secreted Glycoproteins of Marek's Disease Virus and Herpesvirus of Turkeys

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SUMMARY

Hybridomas were formed between mouse myeloma cells and spleen cells from mice immunized with Marek's disease virus (MDV) or with herpesvirus of turkeys (HVT). Three monoclonal antibodies were obtained, two (M26 and M34) from MDV clones and one (H9) from an HVT clone, all of which were specific for cross-reactive membrane antigen (MA) expressed on the surface of cells infected with MDV or HVT. All three antibodies also reacted with MDV- and HVT-specific glycoproteins in the molecular weight (mol. wt.) ranges 54K to 70K (MDV-gp54/70) and 50K to 64K (HVT-gp50/64), respectively. These glycoproteins constitute the putative 'A' antigens which are found in the medium of cultures infected with MDV or HVT. These results suggest that the cross-reactive MA may correspond to 'A' antigen. Pulse–chase experiments using monoclonal antibodies revealed the presence in virus-infected cells of precursor and processed forms of MDV-gp54/70 and HVT-gp50/64 which differ in size. Moreover, by two-dimensional gel electrophoresis we found that MDV and HVT glycoproteins were separated to heterogeneous spots by electric charge as well as mol. wt. The several spots with higher mol. wt. and with more acidic isoelectric points among them were lost by treatment with neuraminidase, suggesting that the processing was, at least in part, due to the addition of sialic acid to the precursor forms. Tunicamycin blocked the surface expression of cross-reactive HVT-MA on HVT-infected cells. Phosphonoacetic acid inhibited both the appearance of HVT-MA on the cell surface and synthesis of HVT-gp50/64, indicating that the MA and secreted glycoprotein were late gene products of the HVT genome.

INTRODUCTION

Marek's disease virus (MDV) is the aetiological agent of Marek's disease (MD), a highly contagious malignant lymphoma of chickens. A herpesvirus of turkeys (HVT) has been successfully used as MD vaccine. All strains of MDV and HVT are antigenically closely related (Chubb & Churchill, 1968; Purchase, 1969; Witter et al., 1970). The serological relationship of virulent MDV, avirulent MDV and HVT is of great interest in relation to the pathogenesis of MD and immunity to this disease. A serological cross-reaction between virus-specific membrane antigen (MA) of cells infected with MDV and HVT has been shown by the immunofluorescence test (Ishikawa et al., 1972). The MA appears to be closely related to the 'A' antigens of MDV and HVT, since these antigens were both lost in some virus preparations (Nazerian, 1973). The 'A' antigen was originally found both in cell extracts and culture fluid of MDV-infected cells by immunodiffusion analysis (Churchill et al., 1969), and was identified as a glycoprotein with a molecular weight (mol. wt.) of 70000 (70K) to 90K (Ross et al., 1973), 44-8K (Long et al., 1975), 52K to 72K (Van Zaane et al., 1982a) or 61K to 65K (Glaubiger et al., 1983). Attenuation of MDV in culture was reported to result in loss of 'A' antigen (Churchill et al., 1969). This finding is of great importance, since MDV becomes attenuated during serial passages in cultured cells...
and the attenuated MDV does not produce MD tumours in chickens (Churchill et al., 1969). However, pathogenic clones of MDV strain JM failed to produce the ‘A’ antigen, while apathogenic HVT produced the antigen (Purchase et al., 1971). In addition, the ‘A’ antigen was detected in the medium of cells infected with an MDV strain that was reported to have lost its capacity for production of ‘A’ antigen (Van Zaane et al., 1982b). These different results could be due to differences in sensitivity of the techniques employed for detection of ‘A’ antigen. Little is known about the immunological and biochemical relationships of MDV and HVT with respect to ‘A’ antigen or MA. Therefore, molecular biological studies on the structure and properties of ‘A’ antigen are required to determine the pathological and immunological significance of the antigen.

Recently, we identified more than 40 MDV-specific polypeptides in chick embryo fibroblasts (CEF) by immunoprecipitation with chicken or duck antibodies purified by affinity chromatography, and found that seven of them were glycosylated (Ikuta et al., 1981, 1983). Most virus-specific polypeptides in cells infected with MDV or HVT possess cross-reactive antigenic determinants and most cross-reactive glycoproteins secreted into the culture medium of MDV- and HVT-infected cells were found to have mol. wt. of approximately 65K (gp65) and 56K (gp56), respectively, and thus may correspond to ‘A’ antigens (Ikuta et al., 1981, 1983).

In the present study, monoclonal antibodies reacting with the surface of cells infected with MDV or HVT were screened by the membrane immunofluorescence (MIF) test and were found to cross-react with MDV and HVT glycoproteins secreted into the medium of virus-infected cultures. The changes of these glycoproteins during serial passages of MDV and HVT in culture were examined by immunoprecipitation with the monoclonal antibodies followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The processing of glycoproteins secreted during MDV and HVT infection from the precursors to processed forms was studied by pulse-chase experiments, and by treatment of the glycoproteins with neuraminidase or tunicamycin (TM).

**METHODS**

*Viruses and cells.* Three MDV strains, BC-1, JM and C2, and one HVT strain, 01, were propagated in primary CEF as described previously (Ikuta et al., 1981). At low passages, the BC-1 and JM strains used had the ability to form typical MD lymphomas in chickens, whereas at higher passages both MDV strains had lost oncogenicity (Table 1; Hirai et al., 1981b). The attenuated C2 strain, C2(A), used here had been used as a vaccine against MD in Japan. The HVT 01 strain, which has a history of more than 60 serial passages in culture, has lost the ability to protect chickens against MD (Konobe et al., 1979). Infection with cell-associated virus was carried out as described previously (Hirai et al., 1980).

**Radiolabelling of cells.** Mock-infected and infected cells in 60 mm-diameter dishes were labelled with 2 ml of minimum essential medium (MEM) containing 1/10th the normal concentration of methionine, 2% dialysed calf serum, and 50 or 100 μCi L-[35S]methionine (1250 Ci/mmol, Amersham International) from 24 to 48 h post-infection. For pulse-chase experiments, cells starved for 30 min in methionine-free MEM were labelled 48 h after infection for 10 min with 200 μCi/ml of [35S]methionine, then washed with phosphate-buffered saline (PBS), and chased in normal medium. For glycoprotein labelling, the cells were incubated with 2 ml of MEM containing 1/10th the normal concentration of glucose, 2% calf serum, and 100 μCi of D-[6-3H]glucosamine hydrochloride (30-3 Ci/mmol, Amersham) from 24 to 48 h post-infection. For TM treatment, the infected cells at 48 h were pre-incubated for 1 h in medium containing TM (Sigma) at a final concentration of 2 μg/ml, and washed with PBS. The cells were then labelled for 30 min with 200 μCi/ml of [35S]methionine in the presence of TM (2 μg/ml).

**Preparation of antisera and monoclonal antibodies.** HVT antibodies were purified by affinity chromatography as described previously (Ikuta et al., 1981) from anti-HVT sera obtained from specific pathogen-free chickens immunized with CEF infected with HVT strain 01 at passage 30 to 35. The method of production and culture of hybridomas and the screening procedures for MDV- or HVT-specific antibodies were described in detail elsewhere (Ikuta et al., 1982).

**Immunoprecipitation and gel electrophoresis.** Mock-infected or infected CEF labelled with [35S]methionine or [3H]glucosamine were solubilized by sonication in lysis buffer [0-5% Nonidet P40 (NP40), 0.5 mM-NaCl, 1 mM-phenylmethylsulphonyl fluoride, 50 mM-Tris–HCl buffer pH 8.0] as described previously (Ikuta et al., 1981). The lysed cells were centrifuged at 65000 g for 1 h and the resulting supernatant fluids were precipitated with mouse monoclonal antibody. The culture medium was centrifuged at 1500 g for 10 min, re-centrifuged at 65000 g for 1 h and the supernatant fluid was mixed with the same volume of 2 x lysis buffer and then precipitated with chicken
Table 1. Oncogenicity of MDV

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>No. of in vitro passages</th>
<th>Oncogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-I</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>-</td>
</tr>
<tr>
<td>JM</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>-</td>
</tr>
<tr>
<td>C2(A)</td>
<td>65</td>
<td>-</td>
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</table>

antibody or mouse monoclonal antibody. For chicken antibody, rabbit anti-chicken IgG (Miles Laboratories) was used to precipitate the antigen–antibody complex as described previously (Ikuta et al., 1981). For monoclonal antibody, rabbit anti-mouse IgG and Staphylococcus aureus Protein A were used to precipitate the antigen–antibody complex as follows: a mixture of 200 μl of the soluble antigen and 10 μl of 10-fold diluted ascites fluid was left overnight at 4 °C. The antibody–antigen complexes were then mixed with 10 μl of rabbit anti-mouse IgG and incubated at 4 °C for 3 h. The immune complexes were treated with 2 to 4 mg of Protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals), and after constant shaking at room temperature for 15 min, the beads were collected by centrifugation and washed twice with lysis buffer and once with 0.01 M-Tris–HCl pH 8.0. Then the precipitates were subjected to one-dimensional SDS–PAGE or to two-dimensional gel electrophoresis. For one-dimensional SDS–PAGE, the immunoprecipitates were dissolved in sample buffer (1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.01% phenol red, 50 mM-Tris–HCl pH 8.2) and subjected to SDS–PAGE (separation gel, 10% polyacrylamide gel, spacer gel, 4% gel) as described previously (Ikuta et al., 1981). For two-dimensional gel electrophoresis, the immunoprecipitates were subjected to isoelectric focusing in the first dimension and SDS–PAGE in the second dimension as described by O’Farrell (1975) and O’Farrell et al. (1977). The immunoprecipitates were suspended in 20 μl 1% SDS containing 100 mM-2-mercaptoethanol according to the method of Van Zaane et al. (1982a). After 1 h at room temperature, the mixture was heated for 3 min at 95 °C, and then treated with 10 μl of 0.5 M-N-ethylmaleimide. After 1 h at 4 °C, 5 μl of 2-mercaptoethanol, 12 μl of Ampholines (13.3% Ampholines, pH 3.5 to 10, and 3.3% Ampholines, pH 5 to 7; LKB) and 10 μl of 20% NP40 were added. Finally, urea was added to a final concentration of 9.5 M. Polypeptides were separated according to charge by pH gradient electrophoresis using 1-6% Ampholines, pH 3.5 to 10, and 0-4% Ampholines, pH 5 to 7. Electrophoresis was carried out for 30 min at 200 V, 14 h at 300 V and 1 h at 600 V. The gels were equilibrated in SDS–PAGE sample buffer (2-3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM-Tris–HCl, pH 6-8) and then subjected to second-dimension electrophoresis under the same conditions as described for one-dimensional SDS–PAGE above. For preparation of fluorograms, the gels were treated with 1 M-sodium salicylate by the method of Chamberlain (1979).

**Immunologicat procedures.** The indirect immunofluorescence test was carried out as described previously (Ikuta et al., 1982). The isotypes of monoclonal antibodies were determined by immunodiffusion with a cell lysate in 0.5% NP40 in PBS as described by Volk et al. (1982). Isotype-specific antisera were purchased from Nordic Immunological Laboratories, (Tilburg, The Netherlands).

**Neuraminidase treatment.** To remove sialic acid from the glycoproteins, the immunoprecipitates were treated with neuraminidase (0.04 unit/ml, type X; Sigma) for 1 h at 37 °C as described by Cohen et al. (1980).

**Phosphonoacetic acid.** PAA was prepared as indicated by Hirai et al. (1981a) and used at 200 μg/ml.

**RESULTS**

**Isolation of monoclonal antibodies to MA cross-reactive with MDV and HVT**

We attempted to isolate monoclonal antibodies to MDV- and HVT-specific MA. Totals of 50 and 14 hybridoma clones producing virus-specific antibodies, prepared from mice immunized with MDV and HVT antigens, respectively, were screened by the MIF test for antibodies to virus-specific MA. Of these, two MDV clones, named M26 and M34, and one HVT clone, named H9, were found to be producing antibodies cross-reactive with MDV-MA and HVT-MA, which were detected on the surface of virus-infected cells at 48 h. Fig. 1 shows examples of MIF detected with M26 antibody. The number of MA-positive cells decreased in MDV-infected
Fig. 1. Membrane antigen-specific immunofluorescence of MDV- or HVT-infected cells with a monoclonal antibody, M26. Uninfected CEF (a), CEF infected with MDV strain BC-1 (b, 17th passage; c, 85th passage), and CEF infected with HVT strain 01 (d, 30th passage) at 48 h post-infection, were treated with 100-fold diluted ascites fluid containing M26 antibody without fixation. After 30 min, the cells were stained with fluorescein isothiocyanate-conjugated anti-mouse IgG. Magnification, x 350.

cells at higher passages. We could not detect any MA-positive cells among cells infected with the BC-1 and JM strains at passage 85 and 57, respectively, whereas both at low and high passages HVT-infected cells were positive for MA. The M26, M34 and H9 antibodies were all found to recognize MA common to MDV and HVT (data not shown).

The antibody titres of the ascites fluids containing these antibodies against acetone-fixed, virus-infected cells are shown in Table 2. The M26 and M34 ascites reacted at high titres against both MDV- and HVT-infected cells but not against uninfected cells. The H9 ascites fluid also reacted at high titres against MDV-infected cells as well as HVT-infected cells. However, it also reacted slightly with uninfected cells (Table 2), but whether it reacts with antigenic determinants of host cellular components is not known at present. All of the immunoglobulin produced by M26, M34 and H9 hybridoma clones was identified by an immunodiffusion test as IgG\(_1\), which reacted weakly with *S. aureus* Protein A (Goding, 1978). Therefore, for immunoprecipitation using these antibodies, the complex of antigen and antibody was mixed with rabbit anti-mouse IgG and then collected with *S. aureus* Protein A.

**Immunoprecipitation of cross-reactive glycoproteins from the medium of MDV- and HVT-infected cultures using monoclonal antibodies reactive with MA**

As shown in the immunoprecipitats from BC-1 strain- or JM strain-infected culture medium at lower passage levels, these being labelled with \[^{35}\text{S}\]methionine (Fig. 2a), M34 antibody reacted with MDV-specific polypeptides with heterogeneous electrophoretic mobility in the mol. wt. region 54K to 70K (54/70K). The polypeptide of 54/70K was less detectable in the medium
Monoclonal antibody to MDV glycoprotein

Fig. 2. Immunoprecipitation of polypeptides from the medium of MDV- or HVT-infected cultures labelled with \([^{35}S]\)methionine or \([^{3}H]\)glucosamine by a monoclonal antibody, M34. Infected or mock-infected CEF were labelled with \([^{35}S]\)methionine (a) or \([^{3}H]\)glucosamine (b and c) from 24 to 48 h after infection. The medium fraction was immunoprecipitated with M34 antibody (a and b) or HVT antibody purified from hyperimmune chicken serum (c) as described in Methods. The immunoprecipitates were subjected to SDS-PAGE. The viruses used were as follows: MDV, BC-1 strain at passage 15 (BC/15), 17 (BC/17), 42 (BC/42), 62 (BC/62) and 85 (BC/85), the JM strain at passage 17 (JM/17), 37 (JM/37) and 58 (JM/58), and the C2(A) strain at passage 65 (C2/65); HVT, strain 01 at passage 30 (01/30), 31 (01/31) and 90 (01/90). The mol. wt. of the polypeptides were calculated by comparison of their mobilities with those of marker proteins in a calibration kit for mol. wt. determination (Pharmacia Fine Chemicals). Numbers on the left and right sides of figures indicate mol. wt. (× 10⁻³) of polypeptides specific to MDV and HVT, respectively. Non-specific polypeptide bands that were also detected in immunoprecipitates of medium of mock-infected CEF are indicated by arrows without a number on the left of (a).

Table 2. Antibody titres of monoclonal antibodies to MDV- and HVT-induced MA against acetone-fixed virus-infected cells

<table>
<thead>
<tr>
<th>Antibody titre*</th>
<th>Ascites fluid</th>
<th>MDV-infected cells</th>
<th>HVT-infected cells</th>
<th>Uninfected cells</th>
<th>Isotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td>M26</td>
<td>40960</td>
<td>10240</td>
<td>&lt;10</td>
<td>IgG₁</td>
<td></td>
</tr>
<tr>
<td>M34</td>
<td>40960</td>
<td>10240</td>
<td>&lt;10</td>
<td>IgG₁</td>
<td></td>
</tr>
<tr>
<td>H9</td>
<td>10240</td>
<td>10240</td>
<td>40</td>
<td>IgG₁</td>
<td></td>
</tr>
</tbody>
</table>

* Reciprocal of highest antibody dilution showing positive fluorescence.
† Determined by immunodiffusion of hybridoma cell lysate and class-specific immunoglobulin antisera as described in the text.

of infected cultures at higher passage levels: at the 85th passage of BC-1, and the 58th passage of JM strain, no polypeptide was found, as in the medium of cultures infected with the C2(A) strain. These results were consistent with the finding of a decrease in the number of MA-positive cells at higher passages as shown in Fig. 1. It is noteworthy that the MDV polypeptide, determined with the M34 monoclonal antibody, was detected in the medium even at the 42nd
and 62nd passages of the BC-1 strain, and the 37th passage of strain JM when the oncogenicity of MDV was lost, as shown in Table 1. The term 'middle passage' level is used for these passage levels of MDV to distinguish from the high passage levels (BC-1 strain, 85th passage; JM strain, 58th passage). The mol. wt. range of the polypeptides in the medium at the middle passage, 52K to 62K (52/62K), appeared to be narrower than that at the lower passage, indicating the absence of higher mol. wt. components. The medium of HVT strain 01-infected cultures at low or high passage levels was tested for immunoprecipitation with M34 antibody. Results showed that antibody reacted with HVT polypeptides with heterogeneous electrophoretic mobilities in the mol. wt. region of 50K to 64K (50/64K) at both passage levels (Fig. 2a). Since bands of the polypeptides indicated by arrows in Fig. 2(a) were also detected in immunoprecipitates of mock-infected culture medium, they seem to be non-specific polypeptides.

Fig. 2 (b) shows that MDV-54/70K, MDV-52/62K and HVT-50/64K were labelled with [3H]-glucosamine, indicating that these polypeptides are glycoproteins. Although the labelling of MDV-52/62K with [3H]glucosamine is not easily seen in Fig. 2(b), the label was significantly detected in the original X-ray film. The proteins were tentatively named MDV-gp54/70, MDV-gp52/62 and HVT-gp50/64, respectively. Using HVT antibody prepared from chickens immunized with virus-infected cells, we detected MDV-gp54/70 and HVT-gp50/64 as the major cross-reactive glycoproteins in immunoprecipitates of the fraction from the medium of MDV- and HVT-infected cells (Fig. 2c). Therefore, MDV-gp54/70 and HVT-gp50/64 reactive with M34 antibody may correspond to the 'A' antigens described previously (Ikuta et al., 1981, 1983).

The heterogeneity in electrophoretic mobility of these glycoproteins could be due to the presence of glycoproteins in several forms: glycoproteins may differ in size of electric charge of the carbohydrate chains, or the monoclonal antibodies may react with a common antigenic determinant present in several polypeptides of different sizes.

When the infected cell lysates labelled with [35S]methionine for 24 h were immunoprecipitated with M34 antibody, small amounts of MDV and HVT glycoproteins were detectable in the virus-infected cells, indicating that these glycoproteins existed predominantly in the medium (data not shown).

When M26 and Hq antibodies were used for immunoprecipitation from the medium of MDV- and HVT-infected cultures, the resulting SDS-PAGE patterns were almost identical with those of the immunoprecipitates with M34 antibody (data not shown), suggesting that the three monoclonal antibodies used here react with the same glycoproteins. However, these antibodies may each react with a different antigenic determinant present in the same glycoprotein.

**Processing of precursors to the products HVT-gp50/64 and MDV-gp54/70**

The processing of virus-specific glycoproteins in the medium of virus infected cultures was examined by pulse-labelling the virus-infected cells at 48 h with [35S]methionine for 10 min and then chasing the labelled cells as shown in Fig. 3 and 4. After each chase period, the cell lysate and medium were treated with M26 antibody and the immunoprecipitates were divided into two parts. One part was treated with neuraminidase to examine whether the processed glycoprotein contained sialic acid, and the other part was incubated without enzyme. The samples were then analysed by SDS-PAGE.

Fig. 3 shows the steps of processing of HVT glycoproteins that reacted with M26 antibody. After 10 min pulse-labelling, 54K polypeptide was detected in the cellular fraction of the HVT-infected culture, but not in the medium. After a chase period of 30 min, two polypeptides, 63K and 54K, were found in the cellular fraction and also in the medium. As found in immunoprecipitates after longer chase periods, secretion of the 63K polypeptide into the culture medium resulted in loss of the label from the cellular fraction. The amount of 54K polypeptide in the cellular fraction appeared to decrease after the beginning of the chase period. Therefore, these results indicate that the 54K polypeptide is the precursor of 63K and is converted to the processed form within 30 min. The 54K polypeptide in the culture medium appeared to be degraded after a chase period of 90 min while the 63K polypeptide was still present. Therefore, the 63K polypeptide may be more stable than the 54K polypeptide in the medium.
Fig. 3. Pulse-chase experiment and neuraminidase treatment of the polypeptides determined with M26 antibody in the cells and medium of HVT-infected cultures. CEF infected with HVT strain 01 at passage 30 was pulse-labelled for 10 min with $[^{35}\text{S}]$methionine at 48 h (0 min chase), washed with PBS, and chased in normal medium for 30, 60, 90 and 120 min. After each period of chase, the cell lysate and medium were immunoprecipitated with M26 antibody and the immunoprecipitates were divided into two parts. One part was treated with neuraminidase for 1 h at 37 °C (+), and the other part was incubated in the absence of enzyme for 1 h at 37 °C (−). The samples were analysed by SDS–PAGE. Numbers on the sides of the figure indicate mol. wt. ($\times 10^3$) of polypeptides.

Treatment with neuraminidase appeared to increase the mobility of the 63K polypeptide slightly. However, the slight changes detected by one-dimensional SDS–PAGE may not be significant.

Fig. 4 shows the processing of MDV glycoproteins immunoprecipitated with M26 antibody from cells infected with the BC-1 strain at passage 17. After pulse-labelling for 10 min, two polypeptides of 61K and 57K were detected in the cellular fraction of MDV-infected cultures. When the infected cells were pulse-labelled with $[^{35}\text{S}]$methionine and chased for 1 h, the cellular fraction gave a faint diffuse band in the region of 64K to 70K (64/70K), which is not easily seen in Fig. 4, in addition to 61K and 57K polypeptides, while the medium fraction gave a diffuse band from 57K to 70K (57/70K). The diffuse band in the sample from the medium fraction after chase periods of 60 min and 120 min could be a mixture of 64/70K, 61K and 57K polypeptides, which were secreted from the infected cells.

When the 57/70K polypeptides in the medium fraction of the MDV-infected culture were treated with neuraminidase, they were found to be converted to two distinct polypeptides of 64K and 58K, but the enzyme did not affect the 61K and 57K polypeptides in the cellular fraction (Fig. 4). These results suggest that the 61K and 57K polypeptides were precursor forms of 64/70K polypeptide. These precursor polypeptides might be processed, at least in part, to the 64/70K polypeptide by sialylation.
Fig. 4. Pulse-chase experiment and neuraminidase treatment of the polypeptides determined with M26 antibody in the cells and medium of MDV-infected cultures. CEF infected with MDV strain BC-1 at passage 17 were pulse-labelled for 10 min with $^{35}$S]methionine at 48 h (0 min chase), then chased in normal medium for 60 and 120 min. After each period of chase, the cell lysate and medium were immunoprecipitated with M26 antibody, and the immunoprecipitates were incubated in the presence or absence of neuraminidase, and then subjected to SDS-PAGE, as for Fig. 3. Numbers on the sides of the figures indicate mol. wt. ($\times 10^{-3}$) of polypeptides.

Heterogeneity of MDV-gp54/70 and HVT-gp50/64 detected by two-dimensional gel electrophoresis

Because of the presence of the precursor and processed forms of similar mol. wt., and possibly because of heterogeneity in their carbohydrate contents, MDV-gp54/70 and HVT-gp50/64 labelled with $^{35}$S)methionine or $^{3}$H]glucosamine for 24 h might form a broad band on SDS-PAGE, as shown in Fig. 2.

To enhance the resolution of the processed molecules, the immunoprecipitates with M34 antibody before and after treatment with neuraminidase were analysed by two-dimensional gel electrophoresis using isoelectric focusing followed by SDS-PAGE (Fig. 5). The medium fractions of virus-infected cultures, which were labelled with $^{35}$S)methionine for 24 h, were
Monoclonal antibody to MDV glycoprotein

Fig. 5. Two-dimensional gel electrophoresis of MDV-gp54/70 and HVT-gp50/64 glycoproteins, before and after neuraminidase treatment, immunoprecipitated from the medium of infected cultures with M34 antibody. CEF mock-infected (a and d) or infected with MDV strain BC-1 at passage 20 (b and e) or HVT strain 01 at passage 30 (c and f) were labelled with [35S]methionine for 24 to 48 h after infection. The culture medium was immunoprecipitated with M34 antibody, as for Fig. 2. The immunoprecipitates, before (a to c) and after (d to f) neuraminidase treatment, were subjected to two-dimensional gel electrophoresis with isoelectric focusing and SDS-PAGE as described in Methods.

used for immunoprecipitation to obtain sufficiently radiolabelled glycoproteins. Both MDV-gp54/70 (Fig. 5b) and HVT-gp50/64 (Fig. 5c) were separated into spots heterogeneous in electric charge as well as mol. wt. In electric charge, MDV-gp54/70 ranged from isoelectric point (pI) 4.0 to 5.6, while HVT-gp50/64 was from about pI 4.0 to 5.8. The mol. wt. of MDV-gp54/70 and HVT-gp50/64 were consistent with those shown by one-dimensional SDS-PAGE (Fig. 2). The patterns were similar to those of the glycoproteins which were pulse-labelled and chased as described in the legends of Fig. 3 and 4 (data not shown). It should be noticed that the spots with higher mol. wt. had a more negative charge. This could be due in part to the addition of sialic acid to the protein. To test this possibility, MDV-gp54/70 and HVT-gp50/64, prepared similarly as for Fig. 5(a to c), were treated with neuraminidase, and then separated by two-dimensional gel electrophoresis (Fig. 5d to f). Neuraminidase treatment resulted in an alteration in charge as well as mol. wt. of both MDV-gp54/70 and HVT-gp50/64. After neuraminidase treatment, several spots with higher mol. wt. and with more acidic pI, which were detected in the sample before neuraminidase treatment, were lost. The spots identified in the sample after neuraminidase treatment appeared to be consistent with the position corresponding to the spots with more basic pI and with lower mol. wt. in sample before neuraminidase treatment. Therefore, the heterogeneity in size and charge of these glycoproteins could be due in part to the
presence of glycoproteins with more heterogeneous carbohydrates which were processed by sialylation.

*Effect of TM on the synthesis of MDV and HVT glycoproteins detected with M26 antibody*

TM has been reported to inhibit the attachment of N-acetylglucosamine to dolichol phosphate, which results in loss of oligosaccharide chains from proteins (Waechter & Lennarz, 1976; Struck & Lennarz, 1977). Therefore, by use of TM it is possible to demonstrate the presence of the unglycosylated forms of secreted virus-specific glycoproteins. Virus-infected cells were labelled at 48 h with [35S]methionine for 30 min in the presence of TM and the cell lysate was immunoprecipitated with M26 antibody and subjected to SDS-PAGE.

Fig. 6 shows that the unglycosylated polypeptides in the presence of TM by MDV- and HVT-infected cells gave single bands at 45K and 46K, respectively. In addition, the unglycosylated MDV polypeptide of 45K was synthesized in cells infected with the BC-1 strain at both low (17th) and middle (42nd) passage levels.

*Effects of TM and PAA on expression of HVT-MA on the surface of virus-infected cells*

The results on MIF shown in Fig. 1 suggested that the glycoproteins determined with the monoclonal antibodies were located on the surface of MDV- and HVT-infected cells. TM has been reported to prevent exposure of HSV glycoproteins on the cell surface (Norrild & Pedersen, 1982). Therefore, using the immunofluorescence test, we examined whether the virus proteins determined with M26 antibody are exposed on the cell surface in the presence of TM. Since it is difficult to obtain sufficient cell-free MDV, but easier to obtain cell-free HVT, we infected the cells with the latter and cultured them in the presence of TM. As cell-free virus, the culture fluid of the HVT 01 strain at passage 90 was used after centrifugation at 1500 g for 10 min, since the culture fluid of cells infected with the 01 strain at high passages contains a large amount of infectious cell-free virus (Konobe et al., 1979). At 48 h post-infection, cells with and without fixation with acetone were examined by the immunofluorescence test using M26 antibody. The results are summarized in Table 3. When TM-inhibited, HVT-infected cells were tested, no cross-reactive MA was observed in unfixed cells, but immunofluorescence was found in the cytoplasm of fixed cells (Table 3, Fig. 7). The strong cytoplasmic fluorescence in the presence of TM appeared in the form of coarse condensed granules, which may correspond to the accumulated unglycosylated polypeptide of 46K seen in Fig. 6. Thus, TM prevented the surface exposure of cross-reactive HVT-MA on the cells, but not the synthesis of the polypeptides determined with M26 antibody.

Next, we examined the effect of PAA on the expression of cross-reactive HVT-MA and on the synthesis of the polypeptides determined with M26 antibody. For this, 200 μg/ml of PAA was added to cells infected with cell-free HVT, since this concentration of PAA completely inhibited the synthesis of HVT DNA in infected cells (Hirai et al., 1981 a). In an immunofluorescence test with M26 antibody, no reaction was observed in either unfixed or fixed cells (Table 3). Therefore, PAA blocked the synthesis of the polypeptides determined with M26 antibody as well as the surface expression of cross-reactive HVT-MA. The result indicates that the precursor and processed forms of HVT-gp50/64 may be late proteins in the HVT replication cycle.
Monoclonal antibody to MDV glycoprotein

Fig. 6. Immunoprecipitation of polypeptides with M26 antibody from MDV- or HVT-infected cells cultured in the presence of TM. CEF infected with MDV strain BC-1 at passage 17 (BC/17) and 42 (BC/42), with strain JM at passage 17 (JM/17), and with HVT strain 01 at passage 30 (01/30) or mock-infected, were preincubated for 1 h in medium containing 2 μg/ml of TM at 48 h, and washed with PBS. The cells were then labelled for 30 min with [35S]methionine in the presence of 2 μg/ml TM. The cell lysates were immunoprecipitated with M26 antibody, and the immunoprecipitates were analysed by SDS-PAGE. Numbers on the right of the figure indicate mol. wt. (×10⁻³) of polypeptides.

Fig. 7. Immunofluorescence of HVT-infected cells cultured in the presence of TM using M26 antibody. CEF on a coverslip were infected with the culture fluid of CEF infected with HVT strain 01 at passage 90, which contained enough cell-free virus (Konobe et al., 1979). After adsorption for 1 h at 37 °C, the cells were cultured in the presence of 2 μg/ml TM for 48 h, and fixed with acetone for 10 min, and then subjected to an immunofluorescence test with 100-fold diluted ascites fluid containing M26 antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG. Magnification × 420.
DISCUSSION

The present studies using monoclonal antibodies clearly showed that the cross-reactive MA components expressed on the surface of MDV- and HVT-infected cells (Fig. 1) and associated with MDV-gp54/70 and HVT-gp50/64, respectively, are secreted into the medium of virus-infected cultures (Fig. 2 to 4). In two-dimensional gel electrophoresis, these secreted glycoproteins appear as a series of spots (more than ten) which increase in charge as they increase in mol. wt.; i.e. the most acidic member of the series was the largest molecule (Fig. 5). These glycoproteins may correspond to the 'A' antigen of MDV and HVT, since the proteins are major virus-specific proteins immunoprecipitated from the culture medium, as described by Churchill et al. (1969). In addition, the mol. wt. of MDV-gp54/70 and HVT-gp50/64 estimated by SDS-PAGE were within the range reported by other investigators (Ross et al., 1973; Long et al., 1975; Van Zaane et al., 1982a; Glaubiger et al., 1983). Previously, Nazerian (1973) observed that viruses that did not produce the 'A' antigen also did not induce the MA, suggesting the similarity or identity of MA and 'A' antigen. We found that the losses of the two antigens during serial passage of the MDV BC-1 and JM strains and in the attenuated C2(A) strain were not coincidental, but possibly due to a result of similarity between or identity of the two antigens. As a middle passage level in culture, when the oncogenicity of MDV was lost, the MDV glycoprotein was still found in the culture fluid (Fig. 2). However, the difference in size of the glycoproteins between MDV at low and middle passages in culture was observed, although unglycosylated polypeptides of 45K were similarly synthesized in cells infected with MDV at low and middle passages in the presence of TM (Fig. 6). Further studies are needed on whether the loss of high mol. wt. components of MDV-gp54/70 at middle passages in culture is associated with loss of oncogenicity.

In the present work, we also studied the processing of HVT-gp50/64 and MDV-gp54/70 by one-dimensional SDS-PAGE of the glycoproteins pulse-labelled and chased (Fig. 3 and 4), and by two-dimensional gel electrophoresis of the glycoproteins labelled for 24 h (Fig. 5), before and after neuraminidase treatment. Pulse-chase experiments revealed that the 54K polypeptide in HVT-infected cells was the precursor of HVT-gp50/64 glycoprotein and that it was processed to a 63K polypeptide, and then secreted into the culture medium. On the other hand, the processing steps for MDV-gp54/70 are not as clear as those of HVT-gp50/64. This may be partly because it is difficult to grow MDV in cultured cells and so the precursor and processed forms of the glycoprotein would not be labelled well. Two distinct polypeptides of 61K and 57K were identified as precursor forms of MDV-gp54/70 glycoprotein, and these precursor polypeptides were processed to the 64/70K polypeptide, and then secreted into the culture medium. However, the reason for the appearance of two precursor forms is not clear. These polypeptides may not have identical amino acid sequences but they are immunologically similar. Differences between these polypeptides could exist in steps for post-translational cleavage or post-translational modification, such as glycosylation, phosphorylation or sulphation.

Two-dimensional gel electrophoresis of HVT-gp50/64 showed that several spots with higher mol. wt. and with more acidic pI were lost after neuraminidase treatment (Fig. 5). These results indicate the presence of sialic acid in the processed form of the glycoprotein. Similarly, it was shown that two distinct precursor forms of MDV-gp54/70 glycoprotein, the 61K and 57K polypeptides, were processed, at least in part, by the addition of sialic acid. If HVT-gp50/64 and MDV-gp54/70 glycoproteins are glycosylated by the binding of an N-acetylglucosamine residue to core polypeptide, their synthesis would be expected to be inhibited by TM. The proteins synthesized in the presence of TM were a 46K polypeptide in HVT and a 45K polypeptide in MDV, as determined with monoclonal antibody (Fig. 6). The finding suggests that the 46K and 45K polypeptides are processed to the precursor 54K polypeptide of HVT-gp50/64 and to the precursor 61K and 57K polypeptides of MDV-gp54/70, respectively, by formation of an N-glycosidic bond. However, the real unglycosylated precursor polypeptide may be degraded in the presence of TM, because unglycosylated proteins are reported to be more susceptible to degradation (Schwarz et al., 1976). The membrane glycoprotein G of vesicular stomatitis virus is glycosylated while its polypeptide chain is being elongated, and when the
glycosylation was artificially blocked with TM, chain elongation of the G polypeptide was incomplete (Rothman et al., 1978). It is, therefore, possible that the 46K and 45K polypeptides observed in the present work are incomplete nascent polypeptides. At present, we cannot exclude any of these possibilities. Structural analyses of the polypeptides synthesized in the presence and absence of TM are required to clarify this problem.

Our results with monoclonal antibodies also showed that TM inhibited exposure of cross-reactive HVT-MA on the surface of HVT-infected cells. Therefore, the unglycosylated HVT polypeptide of 46K detected with the monoclonal antibodies used here could not be transported to the surface of virus-infected cells and appeared to accumulate in the cytoplasm of infected cells.

We found HVT-gp50/64 in the medium of HVT-infected cultures at a high passage level when the ability of the virus to protect chickens from MD was lost. Previous investigators demonstrated that HVT at high passages was not protective because it did not grow in chickens, although it grows well in cultured cells (Konobe et al., 1979; Witter & Offenbecker, 1979). Therefore, our result does not exclude the possibility that the viral glycoprotein is involved in protection against MD. It is noteworthy that chickens vaccinated with purified membranes of HVT-infected cells were protected from MD (Kaaden et al., 1974). The glycoproteins isolated from the membrane fractions of HVT-infected cells were found to give three polypeptide bands in the mol. wt. region of 100K to 120K on SDS-PAGE and inoculation of chickens with these glycoproteins resulted in production of neutralizing antibody and partial protection against MD, suggesting the possible role of these polypeptides in humoral immunity to MD (Wyn-Jones & Kaaden, 1979). From its difference in size, HVT-gp50/64 in the present work appears to be different from the glycoproteins found by Wyn-Jones & Kaaden (1979). Since HVT-gp50/64 and MDV-gp54/70 were expressed on the surface of virus-infected cells, the possible role of these glycoproteins in a cell-mediated response of tumour immunity should be examined.

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REFERENCES


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