Induction of Virus-Neutralizing Antibody by Glycoproteins Isolated from Chicken Cells Infected with a Herpesvirus of Turkeys

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The production of virus-induced proteins in chicken embryo fibroblast cells infected with a herpesvirus of turkeys was studied. It was found that glycoproteins isolated from membrane-rich fractions of infected cells by affinity chromatography using concanavalin A induced neutralizing and precipitating antibody in rabbits and chickens. After analytical electrophoresis, such isolates were found to contain three polypeptide bands of between 100 × 10^2 and 120 × 10^2 molecular weight not present in glycoprotein extracts of uninfected cells, and these polypeptides were further purified by preparative polyacrylamide gel electrophoresis. Inoculation of chickens with purified material also resulted in the production of precipitating and neutralizing antibody, showing that these high-molecular-weight polypeptides play a role in the humoral immunity to Marek’s disease. Challenge of these chickens with virulent Marek’s disease virus revealed that a partial protection was afforded by the inoculated glycoproteins.

Infection of avian cells with Marek’s disease virus (MDV) or turkey herpesvirus (HVT) elicits the production of a number of virus-induced proteins. Soluble antigens in the cytoplasm of infected cells (4) as well as virus-induced proteins in the cellular membranes (3, 5) have been described. The finding that virus-induced proteins prepared from cells productively infected with MDV or HVT were protective in chickens vaccinated against tumor development (6, 8) provided experimental evidence that these proteins contributed to immunity against Marek’s disease. More recently, when lymphoblastoid cells transformed by MDV became available (2, 12), tumor-specific antigens were found to be involved in cell-mediated immune mechanisms, and this helped to create the concept that the immunity against Marek’s disease may be regarded as a two-step mechanism including both virus-induced and tumor-specific antigens (10). In this report we describe the isolation and purification of virus-induced glycoproteins with relative molecular weights between 120,000 and 150,000 from cells productively infected with HVT and the production of neutralizing antibodies in chickens and rabbits.

MATERIALS AND METHODS

Production of crude membrane-rich fraction. Secondary chicken embryo fibroblast (CEF) cells were grown in 880-cm² roller vessels in tissue culture medium 199 supplemented with 2% fetal bovine serum, antibiotics, and insulin (0.4 IU/ml) (14). For maintenance of cultures, the same medium was used, omitting the insulin. Cells were infected 48 h after seeding with 0.5 plaque-forming units of the FC126 strain of HVT (15) per cell, and the cultures were incubated for a further 72 h, when a cytopathic effect involving 70 to 90% of the cells was apparent. They were harvested in the following way: the medium was discarded, and the monolayers were washed twice with phosphate-buffered saline at 37°C. They were drained, and the cells were scraped off the glass with a rubber policeman into about 20 ml of phosphate-buffered saline per vessel and centrifuged at 800 × g; the pellets were resuspended in isotonic reticulocyte standard buffer, pH 7.4 [tris(hydroxymethyl)aminomethane (Tris), 10^{-2} M; KCl, 10^{-2} M; MgCl₂, 10^{-4} M] at 0°C. Clumps were broken up by gentle homogenization with a Dounce homogenizer fitted with a loose pestle. The cells were adjusted to a concentration of 10^7 per ml and were washed once in reticulocyte standard buffer and then swollen in the same buffer diluted 1:10 prepared to pH 8.8. After being allowed to swell for approximately 20 min, they were lysed by the addition of Triton N-101 nonionic detergent (Serva, Heidelberg, Germany) to a final concentration of 0.2% (vol/vol). The detergent was allowed to act for 15 min at 0°C with gentle agitation of the cells, the preparation was then centrifuged at 2,200 × g to remove larger debris, and the resulting supernatant was ultracentrifuged at 100,000 × g for 1 h. The supernatant of this high-speed run was concentrated by ultrafiltration over a PM-10 membrane (Amicon Corp.), the protein-free filtrate being discarded. The material was concentrated to either between 10 and 15 ml or between 80 and 100 ml, depending upon its subsequent treatment.
and dialyzed overnight against concanavalin A (ConA) buffer (Tris, 0.02 M; NaCl, 0.5 M; CaCl₂, 10⁻³ M; MgCl₂, 10⁻³ M). It was stored for not longer than 48 h at 4°C.

Isolation of glycoprotein-rich fraction. Isolation of glycoprotein-rich fractions was achieved by affinity chromatography on ConA-Sepharose (Pharmacia Ltd). One of two methods was used for each batch of crude protein prepared. In the first method, 10 to 15 ml of crude protein was mixed with sufficient ConA-Sepharose to impart a sloppy consistency to the gel and then mixed for 2 h at room temperature. The mixture was placed in a chromatography column, and unbound protein was removed by extensive washing of the gel with ConA buffer until the optical density of the eluate at 280 nm was <0.1. The glycoprotein fraction was eluted with 0.2 M α-methyl-d-mannoside (Sigma Chemical Co.) dissolved in ConA buffer. Fractions were collected until the optical density at 280 nm was again <0.1.

In the second method, less concentrated crude membrane fraction was pumped over washed ConA-Sepharose already packed in a column, the gel then being washed with buffer until all unbound protein was removed; elution of glycoprotein was performed as described above.

The glycoprotein-containing fractions were pooled and concentrated over a PM-10 membrane to between 2.0 and 6.0 ml and stored at -65°C after a sample had been removed for protein determination. Samples were removed at different stages of the above procedure to keep a check on the quantity of glycoprotein recovered as a percentage of the total input protein, since this latter quantity varied considerably over a large number of runs and it was important to know that approximately the same percentage of glycoprotein being recovered each time so that the performance of the ConA-Sepharose could be monitored. Protein determinations were carried out by the method of Lowry et al. (9), modified for Triton-containing fluids by the addition of 50 μl of 20% (wt/vol) sodium dodecyl sulfate to each 150-μl sample, 0.5% (vol/vol) Triton being used as a blank (13).

PAGE. Polyacrylamide gel electrophoresis (PAGE) was used both for analysis of the glycoprotein mixtures and for the preparation of virus-induced glycoproteins of over 100 x 10⁶ molecular weight. Laemmli’s buffers (7) were used. The main gels were either a 8.5% (wt/vol) total acrylamide (Serva, Heidelberg) concentration or gradients of 7.5 to 20% acrylamide. In both types, gels were cross-linked with diallyltartardiamide (Bio-Rad) in a ratio of 1 part diallyltartardiamide to 30 parts acrylamide. A starch gel of 3% total acrylamide cross-linked with bisacrylamide (Serva, Heidelberg) in a 1:38 ratio was used. Samples (protein content, 100 μg) were prepared for electrophoresis by overnight dialysis against water followed by lyophilization and dissolution in 0.1 ml of splitting buffer, pH 7.0, containing 0.005 M Tris, 5% 2-mercaptoethanol, and 2% (wt/vol) sodium dodecyl sulfate. The splitting buffer was allowed to act overnight at 4°C, and the samples were then heated for 4 min at 100°C. After cooling in ice, 10 μl of 10% (wt/vol) bromophenol blue in 30% (vol/vol) glycerol was added, and between 40 and 60 μl of the mixture was applied to the stacker gel. Electrophoresis was carried out at 24 mA until the bromophenol blue had migrated into the main gel, when the current was reduced to 18 mA. The whole run took between 4 and 6 h. Gels were stained for 60 min in 0.25% (wt/vol) Coomassie brilliant blue prepared in a mixture of methanol (50%), acetic acid (10%), and water (40%). Destaining was done in an isopropanol (10%)-acetic acid (10%)-water (80%) mixture.

For preparative electrophoresis, 8.5% acrylamide gels were used throughout. Analytical electrophoresis showed that it would be desirable to study further the glycoproteins of >100 x 10⁶ molecular weight, and preparative electrophoresis was used to separate these glycoproteins from moieties of lower molecular weight. Samples were prepared as for analytical runs except that the quantity of protein electrophoresed was 2.5 mg. Location of the 100-kilodalton area was originally done by running phosphorylase—a marker protein whose molecular weight was 94 x 10⁶—in parallel. In later experiments, a major cellular polypeptide of about 100 kilodaltons was used as an internal marker for the preparative runs. Its position in unaltered gels was established after removal of the entire stacker gel, by cutting off a strip 2.5 cm wide from one end of the gel, staining and destaining it in the conventional fashion, and using the mobility of the stained 100-kilodalton polypeptide relative to bromophenol blue to find the position of the same polypeptide in the unstained major portion of the gel. Having established this point, the gel was bisected along the line of the 100-kilodalton polypeptide, and the part of interest was cut into four or five strips. These were transferred to a Dounce homogenizer with a minimum volume (<5.0 ml) of TEN buffer, pH 7.0 (Tris, 10⁻² M; ethylenediaminetetraacetate, 10⁻² M; NaCl, 0.15 M). The gel was homogenized in ice with loose- and then tight-fitting pestles until completely disaggregated. Sufficient TEN buffer was added to give a loose suspension of the gel beads, and this was set at 4°C overnight for the protein to elute into the buffer. The gel was then centrifuged at 14,000 x g for 30 min, the supernatant was stored at 4°C, and the overnight elution was repeated, using a fresh volume of buffer. The two eluates thus obtained were pooled and concentrated before being assayed and analyzed electrophoretically and by immunodiffusion.

Immunodiffusion tests. Immunodiffusion tests were performed in 1% agarose gels buffered with Tris-hydrochloride to pH 7.2. Six wells surrounding a central seventh one were cut in a hexagonal pattern and filled with approximately 50 μl of reagent, antigens generally being diluted to give equal quantities of protein in each well. Tests were set at room temperature and read after 24 h and again after 48 h. Antisera consisted of immunoglobulin prepared from pooled sera of chickens contact-infected with Marek's disease and shown to contain antibody to MDV antigens.

Neutralization tests. Antibody to crude and purified fractions was prepared in either rabbits or chickens. With the former, three inoculations, each of between 3 and 4 mg, were given intramuscularly at intervals of 2 days, and a fourth inoculation was given by the same route 1 week later. The first three inoculations were given with Freund complete adjuvant. Seven days after the last injection the animals were
bled and the serum was absorbed for 2 h at room temperature with one-fifth the volume of packed CEF cells to remove anti-CEF antibodies; it was then inactivated at 56°C for 30 min. Dilutions of 1/20, 1/40, and 1/80 were prepared in growth medium, and the mixture was incubated for 1 h at 37°C with 50 plaque-forming units of HVT; the mixture was titrated for 70% plaque reduction in plates of secondary CEF. Control samples from preinoculation sera and sera from rabbits inoculated with material prepared from uninfected cells were also included. Foci were counted after 7 days of incubation, and the dilution of antibody needed to reduce the focus count by 70% was calculated. For chickens, 50 μg of protein was inoculated intramuscularly into each of ten 1-day-old chicks; a similar inoculation regime was then used as above, but the animals were not bled until 6 weeks after the last inoculation. Neutralization tests were performed as before. Chicken sera for the neutralization tests were taken before challenge infection. Six weeks after the first inoculation of antigens, chickens were subjected to challenge infection with pathogenic MDV, strain HPRS-16, by intraperitoneal injection of infectious blood diluted 1:10. They were observed for specific clinical and pathological symptoms during an observation period of 20 weeks.

RESULTS

Yield of crude protein from infected cells. Detergent-treated cells, after centrifugation and ultracentrifugation to remove debris and "insoluble" material, yielded between 3 and 4 mg of crude protein per 10^6 cells (approximately); since each batch of cells was generally initiated in about 20 vessels, this gave about 60 mg of crude protein per batch. A corresponding increase in the crude protein yield was obtained when extraction was carried out on batches of more than 20 vessels. Crude protein fractions isolated from virus-infected cells were found not to contain any herpesvirus-specific infectivity, as shown by three blind passages in CEF.

Yield of glycoprotein from ConA binding. Both methods of ConA chromatography gave approximately the same yield of glycoprotein. The results of three typical experiments are given in Table 1. It can be seen that about 75% of the protein applied to the gel was eluted by washing with ConA buffer, and the glycoprotein fraction, comprising about 3% of the total, was eluted with α-methyl mannoside. The gel was regenerated with 5 M MgCl₂, and analysis of the washings showed that a large proportion of the protein remaining on the gel from the previous run was eluted with this salt. Such high salt concentrations, however, resulted in the eluted protein not reacting with the Marek's disease convalescent sera.

Serological analysis. Rabbits inoculated with crude, infected cell extracts developed antibodies to both virus and chicken cell components (Fig. 1). Of four rabbits immunized, three developed strong antibody, whereas that elicited by the third animal was weaker. Simple absorption of the sera with uninfected chicken embryon cells failed to remove all the anti-chicken antibody, though some was removed by absorption of the sera by uninfected chicken cell extract immobilized on a Sepharose 4B column.

It was also clear from the immunodiffusion patterns (Fig. 2) that the ConA-eluted glycoprotein material was serologically active and that the reaction was specific for the virus-induced proteins, although the rabbit serum also contained some nonspecific activity. Heavy precipitin lines were obtained when this positive glycoprotein antigen was reacted with a pool of immunoglobulin obtained from chickens with acute Marek's disease, indicating that this antigen was specific for MDV and its related types.

Table 1. Glycoprotein yield by ConA-Sepharose chromatography

<table>
<thead>
<tr>
<th>Expt</th>
<th>Crude material</th>
<th>Column washings</th>
<th>α-Methyl mannoside eluate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>%</td>
<td>mg</td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>100</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>71</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>100</td>
<td>68</td>
</tr>
</tbody>
</table>

Fig. 1. RIS 1 to 3, Sera from rabbits immunized with infected cell extracts. +VE AG, Glycoprotein extract from infected cells. -VE AG, Glycoprotein extract from uninfected cells. Arrows a and c indicate rabbit anti-chick precipitation lines. Arrow b indicates line produced through interaction of rabbit antibody with virus-induced proteins elicited in the infected cells; this line is seen not to extend between the well containing rabbit serum and that containing negative antigen.
VIRUS-INDUCED PROTEINS IN HVT-INFECTED CELLS

Fig. 2. RIS 4, Serum from rabbit immunized with infected cell extract. RIS G, Serum from rabbit immunized with Con A-eluted glycoprotein material. +VE AG, -VE AG. See legend to Fig. 1. MD CIG, Marek's disease chicken immunoglobulin. CG, Normal chicken globulin. Arrows a and c indicate nonspecific precipitin lines. Arrow b indicates specific precipitin line.

since no line was obtained between these sera and antigen obtained from uninfected cells or between glycoprotein antigen (positive or negative) and normal chicken globulin (Fig. 2). These results thus indicate that such glycoproteins are specific for the inducing virus (HVT) and related to MDV.

Material of over $100 \times 10^3$ molecular weight purified by preparative PAGE were found to be serologically active in immunodiffusion tests (Fig. 3), though this activity was markedly diminished, an observation which correlated with a comparatively low titer of virus-neutralizing antibody (see below).

PAGE. Analysis by PAGE of the glycoprotein material isolated by ConA chromatography showed some differences as well as obvious similarities in the polypeptide patterns produced by infected and uninfected cell extracts (Fig. 4). Three polypeptide bands, all in the molecular weight range $100 \times 10^3$ to $120 \times 10^3$, were detected in glycoprotein material isolated from infected cells which were not present in uninfected cell extracts. The possession of these three polypeptides of such similar molecular weights may indicate that there is one protein present which dissociates slightly during processing for acrylamide gel analysis and appears as three separate bands. It was on these three polypeptides that further purification procedures were concentrated.
On a preparative scale, the major (and common) polypeptide of approximately $100 \times 10^3$ molecular weight was used as an internal marker. Aliquots of 2.5 mg of protein were electrrophoresed, and, after staining a portion of the gel, the location of the 100-kilodalton band was used as a cutoff point for the rest of the gel. By this means a considerable degree of purification of the three polypeptides unique to infected cells was achieved. Between 300 and 500 $\mu$g of protein was usually recovered by this technique.

**Virus neutralization.** The neutralizing activities of sera elicited by inoculation of either rabbits or chickens with crude or purified material are presented in Table 2. Sera against the crude membrane-rich fractions gave adequate neutralizing activity, so it was apparent that this material, at least, contained virus-induced proteins. On the assumption that some, if not all, of the proteins eliciting neutralizing antibody were glycoproteins, such proteins were isolated and inoculated into rabbits, where they induced the production of antibody, of a titer (1/220) approximately the same as that induced by the crude material. Inoculation of 1-day-old chicks with material eluted from polyacrylamide gels resulted in the production of neutralizing antibody, although this was present at a comparatively low titer.

**DISCUSSION**

Our results have demonstrated unequivocally that HVT induces the production of proteins in infected CEF cells, and these are capable of eliciting neutralizing antibody in experimental animals. We consider these proteins to be glycosylated, owing to the facts that they were shown to bind strongly to ConA and that they could be stained with Schiff's reagent (results not shown). Furthermore, we have shown that such infected cells contain a group of at least three polypeptides of between $100 \times 10^3$ and $120 \times 10^3$ molecular weight not present in uninfected cells and that these may be purified by preparative electrophoresis in polyacrylamide gels and used to induce neutralizing antibody in chickens. This confirms and extends the work of Lesnik and Ross (8), who found that comparatively crude membrane extracts of cells infected with HVT induced precipitating antibodies which were also able to protect chickens against challenge with virulent MDV. It is thus quite possible that such virus-induced glycoproteins as we have found in the cell membrane play an important role in the production of humoral immunity and therefore contribute towards protection against Marek's disease. Whether these proteins are only virus induced, or whether they are virus specific, i.e., structural proteins, remains to be determined. Studies by a number of workers, using a variety of techniques (e.g., 1), have shown that MDV and HVT produce antigens in the cell membrane, but it has not yet been definitely established whether these are structural or nonstructural virus proteins. One indication may be that virus neutralization is a function generally associated with structural glycoproteins; our findings would therefore suggest that the glycoproteins isolated are either structural or serologically related to structural proteins. Furthermore, the antigens appear to be common to both HVT and MDV since an appreciable degree of protection was obtained after challenge of the chickens inoculated with the material purified by electrophoresis. Even though the number of chickens used was small, this test provides a useful indication of the possible involvement of these glycoproteins in the induction of humoral neutralizing antibody.

Technically, the isolation and purification of the high-molecular-weight glycoproteins was ac-

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**Table 2. Virus-neutralizing activities of rabbit and chicken sera against crude and purified HVT-induced glycoproteins**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Inoculum</th>
<th>Virus-neutralizing titer (reciprocal)</th>
<th>Marek's disease specific lesions after challenge infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbits 1</td>
<td>Crude cytoplasmic extract</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Crude cytoplasmic extract</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Crude cytoplasmic extract</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Crude cytoplasmic extract</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Glycoprotein fraction</td>
<td>220</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Uninfected cell extract</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chickens 1</td>
<td>Antigens of over $100 \times 10^3$ mol wt prepared by PAGE</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>Ovary tumor</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>Ovary tumor</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>85</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In a control group of ten chickens subjected to challenge infection, none developed Marek's disease specific lesions or Marek's disease tumors, or both, between 5 and 8 weeks postinfection.

* — No lesion observed.

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complished only at the expense of considerable loss in serological activity, measured principally by the comparatively low virus-neutralizing activity of the chicken antisera. This finding, however, is not surprising considering the rigorous treatment of the samples before they were electrophoresed. Whether the antigen retains its activity throughout the electrophoresis, or whether it regains it after removal of excess sodium dodecyl sulfate, is not clear. Isolation by ConA affinity chromatography was found to be the most efficient method, though experiments were also conducted with Lens culinaris lectin in attempts to isolate higher quantities of glycoprotein; these were unsuccessful, however, a maximum of only 1% of the input protein being recovered from the gel after elution. In addition, in all affinity chromatography experiments it was found that lower percentages of glycoprotein were recovered from uninfected cells. These two observations confirm the finding of Ponce de Leon et al. (11) that it is largely protein specific for α-D-mannoside residues which are synthesized after virus infection. Purification by preparative electrophoresis in polyacrylamide gel was found to be adequate for the separation of the glycoproteins of 100 × 10^3 to 150 × 10^3 molecular weight, found only in the infected CEF, from the lower-molecular-weight material. Attempts to purify the polypeptides by other techniques were unsuccessful.

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LITERATURE CITED


