Serological Relationship between
a Pathogenic Strain of Marek’s Disease Virus, its Attenuated
Derivative and Herpes Virus of Turkeys

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SUMMARY

Precipitating antigens present in extracts of chick embryo cells infected with the
HPRS-16 attenuated strain of Marek’s disease virus (att-MDV) were separated by
gel filtration on Sephadex G200 and some of their properties determined. The two
main antigens detected with convalescent MD serum, referred to as ‘B’ and ‘C’
antigens, had mobilities of 0.55 and 0.25 respectively relative to phenol red on
electrophoresis in 7.5% acrylamide gel. The B antigen was relatively stable and of
low mol. wt. in comparison with the C antigen. B and C antigens were in some
instances also detected in culture medium of infected cells, but were distinguishable
from the A antigen, a major glycoprotein antigen released into the culture medium
of cells infected with HPRS-16. The results of immunodiffusion studies suggest that
B antigen is common to MDV and strains of herpes virus of turkeys (HVT) and that
at least 2 antigens (including C) are MDV specific. The A antigen was also com-
mon to MDV and HVT strains. It was noted however that the capacity of HPRS-
16/att to synthesize A antigen was considerably reduced in comparison with HPRS-
16 and HVT strains, and in some preparations the A antigen could not be
detected. Evidence was also obtained for the presence of HVT-specific antigens
associated mainly with the cell fraction.

INTRODUCTION

The serological relationship between Marek’s disease virus (MDV) and virus strains that
can protect against Marek’s disease, such as attenuated MDV and herpes virus of turkeys
(HVT), is of interest in relation to pathogenesis and immunity. Churchill, Chubb & Baxen-
dale (1969) noted three precipitating antigens in cultures of chicken kidney cells infected
with the HPRS-16 strain of MDV. The major antigen present in culture fluids was referred
to as the ‘A’ antigen. Two further antigens ‘B’ and ‘C’ were detected in addition to the A
antigen in extracts of infected cells. It was also noted that the capacity of virus to produce
the A antigen diminished during attenuation by passage in chicken kidney cells and that
this antigen could no longer be detected in cultures infected with HPRS-16/att. Serological
differences have also been reported between MDV and HVT (Purchase, Burmester &
Cunningham, 1971). However, it is not possible to relate these studies to one another and to

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current work because the antigens referred to as A, B and C have not been characterized and they cannot be unequivocally identified solely on the basis of their association with cells or with culture fluids.

In order to study serological relationships further and to evaluate the role of antigens in pathogenesis and in immunity, it is essential to have a nomenclature for the antigens. This should ideally be based on physicochemical properties and on function and must await purification and characterization of the antigens. Some of the properties of the A antigen have recently been described (Ross, Biggs & Newton, 1973). We report here on the separation of other antigens and on the serological relationship between a pathogenic strain of MDV, its attenuated derivative and HVT, using these antigens as references.

METHODS

Cells and media. Chick embryo (CE) and duck embryo (DE) cells were prepared from 11-day-old and 13-day-old embryos respectively. Chick embryos were of the Houghton Poultry Research Station (HPRS) Rhode Island Red (RIR) strain and duck embryos were obtained commercially (Cherry Valley Ltd, Lincolnshire, England). Cultures were initiated in 199 medium + 5% calf serum + 5% tryptose phosphate broth (TPB) and were maintained in medium containing 1 to 2% serum.

Virus. Cell-associated virus was used to initiate infection at all times. Pathogenic and attenuated strains of MDV were HPRS-16 and HPRS-16/att respectively (Churchill et al. 1969). HVT vaccines were obtained commercially from two different manufacturers. The FC226 strain of HVT, obtained originally from Dr R. L. Witter (East Lansing, U.S.A.) was passed four times in CE cells before use. HPRS-16/att was subcultured five times in CE cells before use.

Preparation of antigens. Standard A antigen was obtained from the medium of DE cultures infected with HPRS-16 by precipitation with ammonium sulphate (50% v/v) and was purified essentially as described previously (Ross et al. 1973). The antigen was adsorbed to DEAE Sephadex A50 in 0.01 M-phosphate buffer, pH 7.1, and was eluted with 0.2 M-NaCl in buffer. It was finally purified by gel filtration on Sephadex G200. The purified antigen was heterogeneous in charge as shown by electrofocusing (pI 4.5 to 5.5) and was homogeneous on electrophoresis in 8.5% acrylamide SDS gels. The protein concentration at the end-point of titration of the antigen (highest dilution forming a precipitin line with standard MD antiserum in immunodiffusion tests) was 15 to 30 µg/ml, corresponding to a 50-fold increase in sp. act. over the original antigen.

B and C antigens were obtained from CE cells infected with HPRS-16/att. Cells were grown in the absence of TPB and were harvested 4 to 5 days after infection at 0.1 p.f.u./cell. Infected cells were scraped from roller bottles, resuspended in distilled water at a concentration of 10⁸/ml and disrupted by ultrasonic treatment at 4°C. An equal volume of water-saturated Arcton 113 (I.C.I. Ltd, England) was then added and the mixture homogenized by further ultrasonic treatment. The preparation was then centrifuged at 100,000 g for 2 h and the supernatant fluid used for further work.

Antisera. A single pool of antiserum obtained from a group of Light Sussex chickens naturally exposed to Marek's disease was used. This serum reacted with both MDV and HVT antigens in immunodiffusion tests and is referred to as convalescent MD antiserum (a-MD ser).

MD-specific serum (sp. a-MD ser) was prepared by absorbing convalescent MD antiserum with HVT antigens obtained by ultrasonic disruption of CE cells infected with the
Antigens of MDV and HVT

FC126 strain of HVT. One ml of serum was added to 10 vol. of infected cell extract (10^8/ml) + DNase (1 mg/ml) + NaCl (8 %). The mixture was incubated at room temperature for 1 h and at 4 °C overnight, and was then centrifuged at 100000 g for 1 h. The supernatant fluid was collected and concentrated to the original vol. of serum. Absorbed serum contained excess HVT antigens and reacted only with MD antigens in immunodiffusion tests.

Antiserum against HPRS-16/att (a-att/MD ser) was prepared by inoculating 2-week-old Marek's disease virus and antibody-free RIR chickens with 10^4 p.f.u. of HPRS-16/att intraperitoneally. A second inoculation was given at 6 weeks of age and the birds were bled 10 days after the second inoculation.

HVT antiserum (a-HVT ser) was prepared by inoculating 2-week-old Marek's disease virus-free Brown Leghorn (BrL) chickens with 10^4 to 10^4.4 p.f.u. HVT/chicken in the leg muscle and bleeding the birds after 6 to 8 weeks.

Immunodiffusion tests were carried out in 1 % Difco Noble agar + 8 % NaCl + 0.02 % Na azide. Wells were 5 mm in diam. and the diffusion distance was 3 mm. Results were recorded after 3 days at room temperature. Photographs were taken using indirect illumination.

Immunofluorescence tests were carried out as described previously using convalescent MD serum and FITC-labelled rabbit anti-chicken globulin (Ross et al. 1973).

Polyacrylamide gel electrophoresis was carried out by the method of Davis (1964) as described previously (Ross et al. 1973). In order to identify the positions of antigens by immunodiffusion after electrophoresis, gels were loaded with 200 μg protein having antigenic titres of at least 1/32 as determined by immunodiffusion.

Gel filtration was carried out on Sephadex G200 packed in a 2.5 cm x 30 cm column. Usually, up to 15 mg protein were applied and materials were eluted with PBS + 0.5 M-NaCl at a flow rate of 12 ml/h. Fractions of 2 ml were collected. Mol. wt. were estimated using a calibrated column as described previously (Ross et al. 1973).

Proteins were estimated by the method of Lowry et al. (1951).

RESULTS

Separation of B and C antigens

Antigenic materials with apparent mol. wt. ranging from 56000 to 150000 were recovered after gel filtration of extracts of CE cells infected with HPRS-16/att (Fig. 1). Plainly, a number of antigens were lost during the process since original material contained at least three antigens and only two were consistently recovered (Fig. 2). However, partial separation of the low mol. wt. component (B antigen) from the higher mol. wt. component (C antigen) was possible. Fractions III and IV contained mainly B antigen with only traces of contaminating C antigen. In two of three separations carried out at room temperature using different batches of antigen, only the B antigen was detectable in fractions III and IV when these were tested against 1/2 and 1/4 dilutions of MD antiserum or when undiluted serum was tested against dilutions of antigen. In one experiment, C antigen was present as a contaminant but was no longer detectable after 1/2 dilution of antigen. Accordingly, fractions III and IV are referred to as Bc preparations. Conversely, fraction II contained a higher concentration of C antigen and is referred to as Cb preparation. Comparison of the electrophoretic patterns of the antigenic fractions and the original material applied to the Sephadex G200 column confirmed that some separation of proteins was achieved. At least 13 bands staining with Coomassie blue were resolved on electrophoresis of the original extract, whereas the bands obtained with comparable amounts of proteins from fractions II and III were fewer in number and some were relatively deeply stained, suggesting absence
of some components and selective enrichment of others. A protein of low mobility (Rm 0.25 with respect to phenol red) present in fraction II was not found in fraction III. It is likely that this protein is the C antigen, since immunodiffusion tests revealed two distinct antigens (Rm 0.25 and 0.55) in fraction II (Fig. 3) but only one (Rm 0.55) in fraction III (not shown). Partially purified B antigen was stable on storage at \(-20\,^{\circ}\text{C}\) for several months but the C
**Antigens of MDV and HVT**

Fig. 3. Immunodiffusion test with MD antiserum following polyacrylamide gel electrophoresis of 200 μg of fraction 11 (same material as for Fig. 1). Arrows indicate positions of B and C antigens and of phenol red (PR).

antigen was inactivated under these conditions. This difference might be exploited to eliminate contaminating C antigen from B preparations. Both B and C were resistant to ether (3 extractions/10 min at room temperature).

**MDV and HVT common and type-specific antigens**

The A antigen was identified in cell extracts and in the medium of CE and DE cultures infected with both FC126 and vaccine strains of HVT. It was also identified in medium from CE cultures infected with HPRS-16/att after extensive concentration but not in extracts of infected cells. This anomaly will be discussed later. To test the effect of passage in CE and DE cells on production of antigen, confluent monolayers of CE and DE cells were infected as described in Methods and cultures were passed at intervals of 3 to 4 days. At the end of each pass culture fluids were harvested and antigens were precipitated with ammonium sulphate. Cells were detached from one bottle with trypsin + versene and were used to infect fresh monolayers and for immunofluorescence. Cells from duplicate bottles were scraped, resuspended in distilled water and disrupted by ultrasonication. Antigens were identified using purified MDA antigen and partially purified B and C antigens as references under optimal conditions for precipitation with MD antiserum. For comparable levels of infection, judged by immunofluorescence and c.p.e., the protein concentration at the end-point of titration of the A antigen present in culture medium was comparable in cultures infected with HPRS-16 and HVT vaccine but was much higher in cultures infected with HPRS-16/att (Table 1). This suggests that HPRS-16/att produces less A antigen than the other strains. Fig. 4 shows identity between an antigen detected in the medium and in cell extracts of HVT-infected cultures and purified MDV A antigen, and further that this antigen is unrelated to B and C. Similar results were obtained with vaccine strains of HVT. In order to confirm the A+ character of HVT vaccine viruses, 2-week-old specific pathogen-free (SPF) BrL chickens
Table 1. Effect of passage in CE and DE cells on production of antigens

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<th>Virus/cell/pass no.</th>
<th>c.p.e.*</th>
<th>Immunofluorescence (% cells +ve)</th>
<th>Antigen in culture fluids</th>
<th>Protein at endpoint of titration of A antigen (mg/ml)</th>
<th>Antigens in cell extracts</th>
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<tr>
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<td>B,C</td>
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* Cytopathic effect judged by frequency of rounded cells: + = sparse; ++ and +++ = 50%; ++++ = confluent, = negative.
† Not determined.

kept in isolators were inoculated with 10⁴-8 p.f.u. intramuscularly and their sera examined for the presence of anti-A antibody. Positive responses were obtained in 50% of the chickens 40 days after inoculation (9/18 and 10/19 for the two vaccine strains respectively). The results are specific since 0/7 of the uninoculated chickens developed antibody, and furthermore reactions of identity were obtained when the positive sera and MD antiserum were tested against purified A antigen (Fig. 5).

B antigen was common to all strains (Fig. 4) and was consistently detected in extracts of infected cells and occasionally in culture medium depending on the degree of infection (Table 1). At least one antigen detectable in MD preparations with MD antiserum was not detected in HVT preparations (Fig. 6). This was confirmed using MD-specific antiserum obtained by absorbing MD antiserum with HVT antigens. Fig. 6 shows further that MDV-specific antigens are neither A nor B and suggests that one of them might be C.

Evidence was also obtained for HVT-specific antigens (Fig. 7). This was confirmed by cross absorption (not shown).
Effect of passage in DE and CE cells on production of antigens

Since previous studies suggested that synthesis of A antigen \textit{in vitro} is both virus- and host-dependent (Churchill \textit{et al.} 1969; Ross \textit{et al.} 1973), it was of interest to investigate the effects of passage in DE and CE cells on the capacity of MDV and HVT strains to synthesize A, B and C antigens. Ideally, it would be desirable to infect cultures with equal numbers of p.f.u. at each pass for all the virus strains, and to allow for differences in growth rate between HPRS-16 and the vaccine virus strains. However, this was impracticable and it was necessary to compromise by adjusting the input multiplicity at each pass so as to achieve comparable levels of infection at the end of the pass. Infection was initiated by seeding confluent monolayers of $10^8$ cells with cell-associated HPRS-16, HPRS-16/att and one strain of HVT vaccine using $10^5$, $10^4$ and $10^3$ p.f.u. respectively. Cultures were then passed at intervals of 3 to 4 days by seeding fresh monolayers with infected cells from the previous pass using $10^6$ to $10^8$ in the case of HPRS-16/att and HVT, and with $10^7$ to $10^9$ in the case of HPRS-16 in order to adjust for the lower growth rate of the latter. The results given in Table 1 suggest that the A antigen is synthesized in both CE and DE cells following infection with HPRS-16 and HVT. They indicate further that synthesis of the A antigen, determined by protein concentration at the end-point of titration, is comparable in both CE and DE cells and is unaffected during 10 passages. B and C antigens were also unaffected during 10 passages.

**DISCUSSION**

We have shown that the major antigens present in extracts of CE cells infected with HPRS-16/att are soluble antigens (not sedimented when centrifuged at 100,000 g for 2 h) which differ in size (Fig. 1) and in mobility on electrophoresis in 7.5% acrylamide gels (Fig. 3). The small mol. wt. component referred to as the B antigen was partially separated...
Fig. 5. Agar gel immunodiffusion test showing presence of anti-A antibody in pooled sera from chickens inoculated with HVT vaccines (a-HVT ser). MDA and MDA (1/2) refer to purified HPRS-16 A antigen and 1/2 dilution respectively.

from the larger antigen (C antigen) by filtration using Sephadex G200 and was identified in extracts of CE and DE cells infected with both HPRS-16 and HVT vaccines. It was thus common to all strains tested. C antigen appears to be MDV-specific. Evidence for this was obtained using MDV-specific serum as shown in Fig. 6. Evidence was also obtained for HVT-specific antigens associated mainly with the cell fraction (Fig. 7) although in some instances type-specific antigens were also detected in medium of highly infected cultures. Clearly, antigens other than A antigen may be present in culture medium, and this reinforces the need for purified antigens as references.

MDV and HVT common and type-specific antigens have recently been reported by Von Bülow & Biggs (1974) and may well correspond to the antigens that we have characterized in this study.

The A antigen was also common to all strains tested. Comparison of the capacity of different strains to produce A antigen is complicated by the fact that different strains grow at different rates and cause different degrees of cytolysis. However, for comparable degrees of infection judged by c.p.e. and immunofluorescence, the protein concentration at the end-point of titration was considered to be the most suitable parameter available. On this basis, the production of the A antigen was comparable in cultures infected with HPRS-16 and HVT vaccine strains but was considerably lower in the case of HPRS-16/att (Table 1). Although we have noted A antigen in medium from CE cultures infected with HPRS-16/att, we have been unable to detect it consistently in subsequent experiments and we have invariably failed to detect it in extracts of infected cells. We believe that this anomaly is due to the relatively low concentration of A antigen in cells compared with medium at the time of harvesting the antigens late in infection. This explanation is consistent with the results of preliminary one-step growth experiments which suggest that A antigen is associated with cells early in infection but is released into the medium at the time of exponential growth. This might also explain the failure to detect A antigen in some preparations of cells infected with HVT vaccine (Fig. 4) even when antigen/antibody ratios were varied.
Fig. 6. Agar gel immunodiffusion test of extracts of CE cells (cell Ag) infected with HPRS-16/att (att/MD), FC126 and vaccine strain of HVT (HVT-vac) with antiserum against MDV (a-MD ser), against att/MDV (a-att/MD ser) and with antiserum specific for MDV antigens (sp. a-MD ser). Bc and Cb are fractions obtained from gel filtration of HPRS-16/att antigens. Arrows indicate MDV type-specific antigens. Precipitin lines between a-MD ser and sp. a-MD ser wells are due to reaction between MD antiserum and excess HVT antigens present in sp. a-MD ser.

Although the failure of Churchill et al. (1969) to detect A antigen in preparations infected with HPRS-16/att appear to conflict with our findings, the differences may be attributed to differences in sensitivity of the immunodiffusion test and to the fact that the virus used is uncloned. This reason could apply also to the finding by Von Bülow & Biggs (1974) that HPRS-16/att and passaged HVT were lacking in A antigen. However, our findings agree fundamentally, since we have noted a great reduction in the capacity of HPRS-16/att to synthesize A antigen. Current experiments suggest that continued passage of some strains of HVT may also result in a reduction in their capacity to produce A antigen. These results suggest that variation in the capacity of virus strains to produce A antigen may depend upon alterations produced by passage in vitro and are of interest in the light of recent findings of Heine et al. (1974), who reported that differences in structural polypeptides in strains of herpes simplex virus are confined to a limited number of non-capsid glycoproteins probably associated with the envelope.

At this stage nothing is known of the role of precipitating antigens in pathogenesis and in immunity to Marek's disease. None of the antigens described here has as yet been detected in extracts of tumour cells. Plainly, the capacity of virus to produce A antigen in vitro cannot be used as a marker of pathogenicity. Purchase et al. (1971), Biggs & Milne (1972) and Nazerian (1973) reached similar conclusions. However, since HPRS-16 has a greater capacity for growth in vivo than HPRS-16/att and HVT strains (Phillips & Biggs, 1972) it is possible that quantitative differences in A antigen concentration in vivo may determine the nature and
intensity of the immune response. It is thus conceivable that the failure of HPRS-16/att to induce humoral antibody to the A antigen may be due to insufficient concentration of antigen.

Antigens that have been found to be common to MDV and HVT are likely to be important in immunity, and their use in immunoprophylaxis is under investigation. Furthermore, the isolation and purification of the type-specific antigens detected in this study should be of value in seroepidemiological studies, which are complicated because of cross-reactions between the different strains.

We would like to thank Dr P. M. Biggs and Dr L. N. Payne for much useful discussion and encouragement during the course of this work.

REFERENCES


Antigens of MDV and HVT


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