Characterization of immune responses to baculovirus-expressed equine herpesvirus type 1 glycoproteins D and H in a murine model

D. Tewari, J. M. Whalley, D. N. Love and H. J. Field

Department of Veterinary Medicine, Madingley Road, Cambridge CB3 0ES, U.K., School of Biological Sciences, MacQuarie University, New South Wales 2109 and Department of Veterinary Pathology, University of Sydney, New South Wales 2006, Australia

A murine intranasal infection model for equine herpesvirus type 1 (EHV-1) was used to evaluate immune responses following immunization with insect cells infected by baculoviruses that express EHV-1 glycoproteins. Baculovirus recombinant glycoprotein D (gD) and gH both induced serum antibodies to EHV-1 when measured by ELISA. The gD recombinant also produced a neutralizing antibody response. Protective immunity, determined by accelerated clearance of virus from the target organs in the respiratory tract, was demonstrated in mice immunized with a baculovirus recombinant expressing gD. In addition to the serological response, evidence is presented which shows that cell-mediated responses also play an important role in protection. Both recombinants induced delayed-type hypersensitivity and lymphoproliferation to EHV-1 antigen. The protective effects of T cells were confirmed by adoptive transfer of spleen cells from baculovirus gD-immunized donors to recipients that were challenged with live EHV-1. Depletion of either CD4- or CD8-bearing cells from the gD-immunized donors reduced the ability of the recipients to clear virus from the target organs, although depletion of CD4 cells had a more marked effect.

Introduction

Glycoproteins of herpesviruses, particularly those of herpes simplex virus (HSV), have been studied in considerable detail because of their ability to induce protective immune responses (Spear, 1985; Norrild, 1985). Protection against lethal infections in animals has been demonstrated for a range of recombinant glycoproteins of HSV-1 including gB, gC, gD and gI (Blacklaws & Nash, 1990; Ghiasi et al., 1991a, b, 1992a, b, c, d). In comparison with HSV, relatively little is known about immune responses to the glycoproteins of equine herpesvirus type 1 (EHV-1), an agent that causes respiratory illness, abortion and neurological symptoms in the horse. Genes encoding up to 10 known or predicted glycoproteins have been identified for EHV-1 (Allen & Yeargan, 1987; Telford et al., 1992). Sequence analysis shows that most of the EHV-1 glycoproteins are equivalent to known HSV-1 glycoproteins, including homologues of gC (Allen & Coogle, 1988), gB (Whalley et al., 1989), gH (Robertson et al., 1991) and gD (Audonnet et al., 1990; Flowers et al., 1991; Whalley et al., 1991). A number of studies have demonstrated that protective immunity can be conferred on laboratory animals by EHV-1 glycoproteins; hamsters were protected from EHV-1 infection by passive immunization with monoclonal antibodies (MAbs) to gp13 (gC), gp14 (gB) and gp17/18 (gD; Stokes et al., 1989) and by vaccination with recombinant vaccinia viruses expressing gp13 and gp14 (Guo et al., 1990).

However these animal models suffer from the disadvantage that the pathogenesis of the infection is very different from that in the natural host and the interpretation of the protection experiments remains open to question. A murine model of EHV-1 infection has been developed which overcomes some of these disadvantages. This model has been characterized in great detail, in terms of virus pathogenesis and the immune response produced following intranasal infection with live virus (Awan et al., 1990; Azmi & Field, 1993a, b). The infection by EHV-1 in the murine model was shown to resemble closely that induced by the same virus strain in experimentally infected specific pathogen-free (SPF) foals (Gibson et al., 1992).

The availability of the murine model and the development of various recombinant expression systems provide an opportunity to investigate protective and other immune responses induced by individual EHV-1 glycoproteins in a more accurate model of EHV-1 disease. Recently the characteristics of EHV-1 gD expressed in insect cells using a recombinant baculovirus were described, including the induction of neutralizing antibodies to EHV-1 and to the closely related respiratory pathogen EHV-4 (Love et al., 1993). A
baculovirus construct has also been generated which expresses EHV-1 gH (McGowan et al., 1994). In this report we describe an evaluation of the immune response to and protective efficacy of these two baculovirus-expressed recombinant EHV-1 glycoproteins following the challenge of mice with live virus.

**Methods**

**Mice.** Three-week-old female BALB/c mice were obtained from Bantin and Kingman. Mice were used within 7 days of arrival.

**Virus and virus assays.** Strain AB4 of EHV-1 was grown at an m.o.i. of 0.01 p.f.u./cell in rabbit kidney (RK-13) cells and assayed by plaque titration. Virus was purified from supernatant fluid on a potassium tartrate gradient as described previously (Tewari et al., 1993). The wild-type baculovirus Autographa california nuclear polyhedrosis virus and recombinant baculoviruses AcNgiD (expressing baculo-gD; Love et al., 1993) and AcNgiH (expressing baculo-gH) were grown in Spodoptera frugiperda IPLB-Sf21-AE cells. These were harvested 48 h after infection at an m.o.i. of 10 p.f.u./cell.

**Mouse immunization and challenge experiments.** Mice were immunized by subcutaneous (s.c.) inoculation with two doses, 15 days apart, of 2 x 10⁶ recombinant virus-infected cells (baculo-gD or baculo-gH). Control mice were inoculated with wild-type virus-infected cells. Additional control mice were not immunized. Fifteen days after the second injection of antigen, immunized and control mice were challenged with 1 x 10⁶ p.f.u./mouse of EHV-1 AB4 intranasally, as described previously (Awan et al., 1990).

To determine the effect of immunization on virus clearance, groups of 18 mice were challenged. Groups of four wild-type-, baculo-gD- and baculo-gH-immunized mice were tested for the presence of virus at 1, 3 and 5 days post-challenge. Groups of three unimmunized control mice were tested at each time point.

**Virus clearance assay.** Lung and nasal turbinate specimens were collected from each of the groups of mice killed after 1, 3 and 5 days following infection and virus titres determined by plaque titration using RK-13 cells.

Groups of 12 mice were used for analysing the virus-specific immune responses to baculo-gD, baculo-gH or wild-type virus. Sera from five mice in each group were pooled for virus neutralization and immunoblot assays and ELISA; the same mice were used subsequently for additional tests.

**ELISA.** This assay was performed as described by Azmi & Field (1993a, b) with some modifications. Briefly, twofold dilutions of test sera were added to plates coated with an optimum dilution (1:200) of purified virus antigen grown in RK-13 cells. The specific binding of the antibody was detected using horseradish peroxidase (HRP)-conjugated immunoglobulins to mouse IgG (Dako) as the secondary antibody and o-phenylenediamine (Sigma) as the substrate. The absorbance was read in dual wavelength mode (492 to 690 nm) using an ELISA Reader (Titertek). The test serum dilution was considered positive if its absorbance value was greater than or equal to twice the average value of the negative control serum. Hyperimmune and normal mouse sera were used as the positive and negative controls respectively.

**Virus neutralization assay.** Dilutions (100 µl) of heat-inactivated (56°C for 30 min) serum were added to 100 µl suspensions of EHV-1 (1 x 10⁴ p.f.u./ml) and incubated at 37°C for 30 min. Following incubation, 100 µl aliquots were added to duplicate wells of RK-13 cells grown in 24-well plates. Overlay was added as for the standard plaque assay and the plates were incubated for 3 days. The plaques were then counted. The serum dilutions that resulted in a 50% reduction in plaque number were taken as the serum neutralizing titres.

**Immunoblot assays.** These were carried out according to published methods (Tewari et al., 1993) using purified EHV-1 AB4 grown in RK-13 cells and baculovirus glycoprotein recombinants grown in insect cells. Polypeptides were separated by 10% SDS-PAGE using a Bio-Rad Mini-PROTEAN II electrophoresis apparatus. Markers (Bio-Rad) were included for determining the relative Mr's. For probing the blots, baculovirus-immune serum was used at a 1:50 dilution and infected-fetal calf serum was used at a 1:40 dilution. MAB 20C4 to gD (provided by Dr George Allen, University of Kentucky, Lexington, Ky., U.S.A.) was used at a dilution of 1:800. The secondary antibodies were HRP-anti-mouse IgG (Dako) at a 1:800 dilution for mouse serum and HRP-rabbit anti-horse IgG (Nordic) at a 1:300 dilution for foal serum.

**Delayed-type hypersensitivity (DTH) test.** Groups of four mice were inoculated with baculo-gD, baculo-gH or wild-type virus as described above and were tested 15 days after the second immunization. A skin test was performed in the ear pinnae according to published methods (Nash et al., 1980) and recently described for EHV-1 (Azmi & Field, 1993a) using purified, heat-inactivated EHV-1 (10⁶ p.f.u. pre-inactivation) grown in RK-13 cells as the challenge antigen. Briefly, antigen (20 µl) was inoculated intradermally into the left ear pinna of each mouse and the ear thickness was measured with a micrometer screw gauge. A similar quantity of uninfected cell lysate was inoculated into the right ear pinna as control. Skin thickness was measured at 0, 24, 48 and 72 h post-inoculation.

**Lymphocyte stimulation test.** This assay was carried out using spleen lymphocytes. Single-cell suspensions were prepared from the spleens of three mice in each of the three immunized groups (baculo-gD, baculo-gH and wild-type) and baculo-gH-immunized mice were tested for the presence of virus at 1, 3 and 5 days post-challenge. Groups of three unimmunized control mice were tested at each time point.

**Depletion of spleen cell populations.** Depletion of T cell subpopulations in donor mice was carried out as described previously (Azmi & Field, 1993b) using MAbs YTS 191.1 (anti-CD4) and YTS 169.4 (anti-CD8) provided by Dr A. A. Nash (Department of Pathology, Cambridge University, U.K.). Nine mice were immunized as above and were tested 15 days after the second immunization. A skin test was performed in the ear pinnae according to published methods (Tewari et al., 1993) using purified, heat-inactivated EHV-1 antigen (1 µg/well). Control wells received medium alone or uninfected RK-13 cell lysate. For stimulation studies, cells were pulse-labelled (after 4 days incubation) with 1-0 µCi of [3H]thymidine (2.0 Ci/mmol) and were further incubated for 18 h. The cells were collected onto filter mats using a semi-automatic cell harvester (Titertek). Results were expressed as mean c.p.m. of the triplicate cultures.
Statistical analysis. The logarithmic virus titres in murine tissue samples were compared by analysis of variance and differences tested by the Scheffe F-test.

Results

Protective immunity

The protective effect of baculovirus products was demonstrated by measuring virus titres in the lungs (Fig. 1a) and nasal turbinates (Fig. 1b) after challenge with EHV-1. Notably, immunization of mice by baculo-gD resulted in a reduction of EHV-1 titres to undetectable levels in the lungs and nasal turbinates by 5 days post-challenge. Analysis of these data also showed significant

<table>
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<tr>
<th>Immunizing agent*</th>
<th>Mean serum IgG ELISA titre</th>
<th>Mean neutralizing antibody titre</th>
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<tr>
<td>gD</td>
<td>1/6400</td>
<td>1/40</td>
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<tr>
<td>gH</td>
<td>1/6400</td>
<td>≤1/10</td>
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<tr>
<td>Wild-type (AcNPv)</td>
<td>≤1/50</td>
<td>≤1/10</td>
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<tr>
<td>Live EHV-1†</td>
<td>1/80</td>
<td>≤1/8</td>
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* Mice were immunized twice, 15 days apart, and analysed for antibody titres 15 days after the second injection. Data are from pooled sera of groups of five mice.
† Antibody titres were determined in a separate experiment in sera obtained from groups of four mice, 3 weeks post-inoculation with live EHV-1 (3 × 10⁶ p.f.u.) intranasally.
reductions in virus titres in baculo-gD-immunized mice compared with wild-type-immunized mice after 3 days. However the baculo-gH-immunized mice showed no consistent decrease in virus titres and the reduction compared with the wild-type-immunized controls reached significance after 5 days in nasal turbinates only.

There were also reductions in the virus titre in the lungs of wild-type-immunized mice compared to unimmunized control mice. Although the effect was significant at one time point only, day 5 in the lungs (Fig. 1a), a similar trend with wild-type immunization has been observed in subsequent experiments.

Serological responses

When analysed by ELISA, mice immunized with baculo-gD and baculo-gH had high serum EHV-1 IgG antibody levels (1/6400), and mice infected with EHV-1 developed a low antibody titre (1/80; Table 1). Neutralizing antibodies were demonstrated only in sera from mice immunized with baculo-gD. Neither EHV-1 virus inoculation nor baculo-gH resulted in detectable neutralizing antibody.

In immunoblotting experiments, sera from baculo-gD-immunized mice recognized a single band at 58K in EHV-1 polypeptide preparations. This band was also recognized by MAb 20C4. However sera from baculo-gD-immunized mice recognized three gD-specific bands (56K, 52K and 48K) in preparations of baculo-gD-infected insect cells. The identification of these bands as gD was confirmed in this and earlier experiments using MAb 20C4 (Love et al., 1993). The three bands are likely to be products of the post-translational processing of the gD molecule. Serum from an SPF foal, infected experimentally with EHV-1, identified two of the three polypeptide bands corresponding to 56K and 52K in the baculovirus-gD recombinant (Fig. 2).

The same EHV-1-infected SPF foal serum, when tested against the baculovirus recombinant expressing gH, recognized a polypeptide at 115K that has previously been observed during characterization of this recombinant (McGowan et al., 1994). The serum from baculo-gH-immunized mice identified a similar band in a recombinant baculovirus expressing gH (Fig. 3). It also recognized (weakly) a polypeptide from purified EHV-1 at an M<sub>c</sub> corresponding to gH. The weakness of the reaction was probably due to the relatively small amount of gH present in EHV-1 antigen preparations.

Cell-mediated responses

Mice immunized with baculo-gD or baculo-gH showed strong DTH responses when tested with EHV-1 whole virus antigens (Fig. 4). The increase in skin thickness compared with the control was maintained for 48 to 72 h in baculo-gD-immunized mice and somewhat less in the case of baculo-gH-immunized mice. However the difference in the response to the two recombinants was not significant (Fig. 4). Both baculo-gD- and baculo-gH-immunized mice also responded to EHV-1 antigens in a proliferation assay, indicating a specific T cell response (Fig. 5).

Transfer of spleen cells from baculo-gD-immune donors to naive recipient mice resulted in the transfer of
Binant product, although it also induced high antibody response, did not reduce virus titres in lung tissues. Some levels in ELISA and a lymphoproliferation and DTH than control mice. In contrast, the EHV-1 gH recombinant following challenge of baculo-gD-immunized mice with live (nasal turbinates and lungs) at a significantly faster rate EHV-1, mice cleared virus from the major target tissues (nasal turbinates and lungs) at a significantly faster rate than control mice. In contrast, the EHV-1 gH recombinant product, although it also induced high antibody levels in ELISA and a lymphoproliferation and DTH response, did not reduce virus titres in lung tissues. Some effect was observed in nasal turbinates. Furthermore baculo-gH did not induce detectable neutralizing antibody.

Both recombinants induced antibody levels in ELISA of significantly higher magnitude than those induced by inoculation of live EHV-1 (Field et al., 1993). This may reflect the route and frequency of antigen presentation, and/or a suppressive effect of live virus on the immune system. This latter possibility is currently under investigation. It is also noteworthy that the recombinant products were derived from EHV-1 strain HVS25A (Sabine et al., 1981) and offered protection against strain AB4. This observation is consistent with the available information on the amino acid sequences of the glycoproteins of these EHV-1 isolates, which indicates considerable homology (Telford et al., 1992; Robertson et al., 1991; Flowers et al., 1991; Audonnet et al., 1990; Whalley et al., 1991).

The results obtained here with the baculo-gD and baculo-gH recombinant products as immunogens in the EHV-1 murine model are consistent with previous data on the ability of the gD and gH homologues in other alphaherpesviruses to induce protective immune responses. A large body of evidence has shown that HSV-1 gD presented in a variety of forms elicits the strongest protective immune responses of any of the glycoproteins (Chan, 1983; Berman et al., 1985; Blacklaws et al., 1987; Martin et al., 1987). The gD homologue of pseudorabies virus, gp50, protects pigs against lethal challenge (Marchioli et al., 1987). HSV-1 gD expressed in insect cells by recombinant baculoviruses induced protective effects in mice challenged with HSV-1 (Krishna et al., 1989; Ghiasi et al., 1991a). Conservation in the function of the gD homologue in EHV-1 has also been indicated by the blocking of virus penetration by a MAbs to EHV-1 gD (Whittaker et al., 1992).

The presence of neutralizing epitopes on EHV-1 gD was revealed by the generation of a number of neutralizing MAbs (Allen & Yeargan, 1987; Whittaker et al., 1992) and by the use of synthetic peptides to identify a stretch of 19 amino acids near the N terminus of the polypeptide which induces neutralizing antibodies (Flowers & O’Callaghan, 1992). The data presented here, along with the observations on passive protection conferred by EHV-1 gD MAbs (Stokes et al., 1989), suggest that humoral immune responses have a role in protection against EHV-1 infection in these models. In the present experiments, it is notable that EHV-1 gH failed to induce neutralizing antibodies and did not markedly affect the virus clearance during the EHV-1 infection. This contrasts with an earlier report for HSV-1 gH, where a baculovirus recombinant product induced a neutralizing response, but did not protect against the lethal effects of HSV-1 in mice (Ghiasi et al., 1992a).

**Discussion**

In this series of experiments, we have shown that mice immunized with a recombinant baculovirus expressing gD of EHV-1 produced high levels of IgG in ELISA and neutralizing antibody to EHV-1, together with DTH and T cell-proliferation responses to EHV-1 antigen. Following challenge of baculo-gD-immunized mice with live EHV-1, mice cleared virus from the major target tissues (nasal turbinates and lungs) at a significantly faster rate than control mice. In contrast, the EHV-1 gH recombinant product, although it also induced high antibody levels in ELISA and a lymphoproliferation and DTH response, did not reduce virus titres in lung tissues. Some protection, producing a significant reduction (P < 0.01) in virus titre in the lungs and nasal turbinates of EHV-1-challenged recipients after 5 days compared with mice immunized with wild-type baculovirus. However when either CD4 or CD8 cells were depleted from donor mice immunized with baculo-gD, the clearance of virus from lungs and nasal turbinates of naive recipients was impaired significantly (Fig. 6; P < 0.01).

**Fig. 6.** Protective effects in the lungs (a) and nasal turbinates (b) 5 days after adoptive transfer of whole immune spleen cells (1 x 10⁷), and in vivo-depleted CD8 cells and CD4 cells from donor mice. Donor mice were immunized twice with 2 x 10⁷ baculovirus-infected cells/mouse 15 days apart and challenged 15 days after the second immunization with 5 x 10⁶ p.f.u./mouse intranasally. Virus titres were determined following the challenge. Mean values are from groups of three mice. Vertical bars represent S.E.M. Pairs of bars marked with the same symbols were found to be significantly different from each other (P < 0.01).
However our results are similar to earlier findings that no neutralizing antibody response was induced by vaccinia-virus-expressed gH, a result which was attributed to differing behaviour of gH during expression in mammalian cells, in the absence of an accompanying gL molecule (Blacklaws & Nash, 1990; Hutchinson et al., 1992). In our experiments, the recognition of a 115K form of EHV-1 gH from insect cells by both equine and murine sera suggests that the processing of the gH molecule is similar to that occurring in normal EHV-1 infection. However it might differ in conformation when expressed along with gL and it will be of interest to investigate the protective response to coexpressed EHV-1 gH and gL in the murine infection model.

Notwithstanding the antibody responses, a role for cell-mediated immunity in the EHV-1 murine model was also demonstrated in the present experiments and this supports previous observations in one of our laboratories that T cells are important in immunity to EHV-1 (Azmi & Field, 1993a,b). Both gD and gH recombinants induced DTH and lymphoproliferative T cell responses, although the responses to the baculo-gH recombinant were somewhat lower than those to baculo-gD. The factors mediating protection in gD-immunized mice were characterized by adoptive transfer of gD-immune spleen cells. Depletion of either CD4- or CD8-bearing T cell subpopulations from immune donor gD mice and transfer to recipients caused an impaired reduction in EHV-1 titre as measured by virus clearance from respiratory tissues. CD4 cells seemed to be more important in this. Using a similar EHV-1 murine model but with live EHV-4 as the immunogen, Azmi & Field (1993b) also reported that the protection was conferred by adoptive transfer of spleen cells. However in those experiments CD8-bearing cells were reported to be more important in effecting virus clearance. Experiments are currently in hand to define mechanistically these observations. Further attempts are now being made to define immunodominant cytotoxic and helper T cell gD epitopes in mice that may serve as potential vaccine candidates for horses in the future.

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