Cell-mediated antiviral response to equine herpesvirus type 1 demonstrated in a murine infection model by means of adoptive transfer of immune cells

M. Azmi and H. J. Field*

Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, U.K.

Protection against equine herpesvirus type 1 (EHV-1) infection in a mouse model has been studied by means of delayed-type hypersensitivity (DTH) and adoptive transfer of immune spleen cells. Mice were found to develop a positive DTH response to EHV-1 antigen which was sustained for several months after primary inoculation. The response was found to cross-react with EHV-4-derived antigen. Immune cells (from mice primed with live or heat-inactivated EHV-1) conferred an enhanced DTH response on recipients; however, only the immune cells that were previously primed with live EHV-1 gave protection against re-infection. The degree of protection was also dependent on the number of spleen cells transferred. Immune cells from mice primed with heat-inactivated EHV-1 appeared to enhance virus replication following subsequent inoculation. The serum antibody response to EHV-1 appeared to be slightly suppressed in recipients of spleen cells from mice primed with either live or heat-inactivated virus. These results support the important role for cell-mediated responses in protective immunity to EHV-1 and provide clues to the nature of protection and immunopathology in the natural host.

Equine herpesvirus type 1 (EHV-1) causes an important infection in horses associated with a variety of clinical problems including respiratory distress, abortion (Bryans & Allen, 1989) and neurological signs (Charlton et al., 1976; Chowdury et al., 1986). The serologically related but distinct virus, EHV-4, also causes a common infection and both EHV-1 and EHV-4 establish latent infections in horses from which they may be reactivated. Commercial vaccines for EHV-1 are available; however, the protection conferred may be incomplete and short lived. Disease has been reported in animals that have been vaccinated (Allen & Bryans, 1986) or previously infected naturally (Grandell et al., 1980). Thus the epidemiology of EHV and the role of immunoprophylaxis are complex and poorly understood.

Much attention has been given to the humoral response to EHV-1 and the role of neutralizing antibody in protection has been investigated in the horse (Allen & Bryans, 1986; Thompson et al., 1976) and in a hamster infection model (Stokes et al., 1989). The importance of cell-mediated responses in immunity to EHV-1 has also been shown in hamsters (Sentsui et al., 1991). The hamster infection model, however, has several features that differ markedly from those seen in the natural disease. A murine model of EHV-1 infection was established using intranasal (i.n.) inoculation and the pathogenesis of the infection in this model has features which more closely resemble the natural infection. These features include respiratory sites for virus replication, viraemia, latency and abortion (Awan et al., 1990, 1991; Field et al., 1992).

In a separate study (M. Azmi & H. J. Field, unpublished) we will report the humoral responses to EHV-1 in the murine model. We demonstrated that EHV-1 infection resulted in protective immunity and reduced viral replication in the respiratory tissues following secondary infection. The protection in the upper and lower respiratory tissues was found to vary with the route of immunization and also protection did not correlate well with antibody levels suggesting that cell-mediated responses may be more important for protection.

The cellular responses in mice to other infections are well understood and the murine model therefore presents a suitable one for the investigation of cell-mediated responses to EHV-1 infection. In the present study we present direct evidence for the important role of cell-mediated immunity (CMI) in establishing protective immunity to EHV-1 infection in the murine i.n. inoculation model and we speculate on the likely importance of these mechanisms in the natural host.

The virus employed was EHV-1 strain AB4 (Awan et al., 1990). Virus working stocks were grown in rabbit kidney (RK-13) cells (or Vero cells) as previously described (Awan et al., 1990) and titrated by a plaque assay in RK-13 cells. In some cases virus inocula were heat-inactivated at 56 °C for 30 min.

Female BALB/c mice were inoculated i.n. at 4 to 5
weeks old with $10^6$ to $10^7$ p.f.u. of EHV-1 (in 40 μl inoculum). When appropriate, uninfected RK-13 cell lysate was used for mock infection of control mice. Mice were killed at various times post-inoculation (p.i.). The whole lungs and turbinate bones were homogenized and tested for the presence of virus ($\log_{10}$ p.f.u. per organ) by titration in RK cell monolayers.

Virus was purified to produce target antigen for the ELISA, for testing the delayed-type hypersensitivity (DTH) response and for the preparation of hyperimmune sera. Infected cell suspensions were sonicated for 3 min and centrifuged at 3000 r.p.m. for 10 min. The supernatant and the cell-free virus supernatant were subjected to ultracentrifugation at 28000 r.p.m. for 2 h at 4 °C. The resultant pellet was collected in a small amount of TNE (Tris, NaCl, EDTA) buffer with repeated pipetting to break the pellet, and then sonicated for 30 s. The virus suspension was gently layered on top of a 60 ml 10 to 50% potassium tartrate linear density gradient in TNE buffer. The gradient was centrifuged at 20000 r.p.m. for 3 h at 4 °C. The white opalescent band was carefully aspirated from about the middle of the gradient. The virus suspension was further diluted 1:4 in TNE buffer before being subjected to centrifugation at 28000 r.p.m. for 1 h at 4 °C. The pelleted virus was resuspended in a small amount of TNE buffer, titrated by means of plaque assay and stored at −70 °C.

For the preparation of anti-EHV-1 hyperimmune sera, BALB/c mice were inoculated subcutaneously (s.c.) with $10^7$ p.f.u. purified EHV-1 (inactivated at 56 °C for 30 min and emulsified with Freund’s complete adjuvant) grown in RK-13 cells. Another three inoculations were given at 2 week intervals using Freund’s incomplete adjuvant. Sera were collected 14 days after the final booster (using live virus without adjuvant), pooled and stored at −20 °C. Antibody titres were determined by means of ELISA. Preimmune or normal mouse sera were collected from uninfected mice. Donor spleen cells were obtained from syngeneic BALB/c mice which had been inoculated i.n. 3 weeks previously with $5 \times 10^6$ p.f.u. live EHV-1 or with the same amount of heat-inactivated virus. Groups of mice were inoculated i.n. with uninfected RK-13 cell lysate and these were used as a source of control donor cells. Twenty-one days after the priming inoculation, the spleens were removed aseptically and pooled before being gently homogenized. The cell suspensions were filtered through fine sterile muslin gauze and centrifuged at 1500 r.p.m. for 10 min. The cells were treated with distilled water for 15 s to lyse erythrocytes. The osmotic balance was then restored in RPMI-1640 medium and the cells were washed twice. Approximately 5 ml samples of the cell suspensions (containing $10^7$ cells) were added and allowed to adhere to Petri dishes by incubation at 37 °C for 1 h. The adherent cells consist of macrophages. Non-adherent cells (mostly lymphocytes) were harvested and pooled. The cell suspensions were centrifuged at 1500 r.p.m. for 10 min and resuspended in a small volume of RPMI. The number of cells and their viability were determined by counting in a haemocytometer using the trypan blue exclusion method. The concentration of the cells was adjusted in RPMI as required for inoculation. The viability was > 95%. Two-hundred μl of the cell suspension was injected into the tail vein of recipient mice. Control mice received spleen cells from the mice inoculated with uninfected RK cell lysate. Virus challenge inoculations were carried out several hours later on the same day of spleen cell transfer.

An antigen was prepared as above except that virus was grown in Vero cells to minimize contamination with cross-reactive cellular antigens. Twenty μl of the inoculum, containing $10^6$ erstwhile p.f.u. EHV-1 (heat-inactivated), was inoculated intradermally into the left ear pinna of mice under general anaesthesia. A similar quantity of mock-infected cell lysate suspension was also inoculated at the same time into the right ear pinna as a control. Mice primed with uninfected cell lysate were inoculated with virus antigen as an additional control. All inoculations were carried out in quadruplicate. The ear skin thickness was measured at daily intervals with an engineer’s micrometer screw gauge. The details of this technique have been previously described (Nash et al., 1980).

An ELISA was developed to detect antibodies reactive with EHV-1 antigens. Briefly, twofold serial dilutions of test sera were added to antigen-coated 96-well plates, then allowed to react with a goat anti-mouse (IgG) antibody conjugated to horseradish peroxidase, using 2,2’-azino-bis(3-ethylbenzthioline-6-sulphonic acid) (Sigma) as the substrate. The absorbance was read at dual wavelength mode absorbance 405 to 492 nm using a Titertek Multiskan multichannel photometer (Flow Laboratories). Antibody titres were determined graphically. This enabled the values of $\log_{10}$ dilution to be read from the curve that corresponded to an absorbance value $\geq$ the mean of eight wells of preimmune sera (±3 S.D.). As a positive control, hyperimmune mouse serum was included on each plate and preimmune or normal mouse sera were used as negative controls. The statistical significance of differences between groups of data was determined using the two-tailed Student’s unpaired t-test.

Mice were inoculated with EHV-1 i.n. or intravenously (i.v.) and re-inoculated by the same route at 2 months. After a further 2 months, a DTH response to EHV-1 antigen was measured. One day after the test injection a positive response to EHV-1 antigen was shown with a greater magnitude in mice inoculated i.n. compared to
Fig. 1. DTH response in mice previously inoculated i.n. or i.v. with live EHV-1. The mice were inoculated either i.n. (■) or i.v. (□) with $2 \times 10^6$ p.f.u. EHV-1 or given uninfected RK cell lysate (▲). To test for DTH response, mice were inoculated 2 months later intradermally into ear pinnae with (a) EHV-1 or (b) EHV-4 antigens. Data points represent mean with S.D. ear thickness increase from groups of four mice.

those inoculated i.v. (Fig. 1a). However, the difference was not significant ($P > 0.05$) and not apparent on day 2 after antigen inoculation. The mice were also found to respond to an antigen preparation derived from EHV-4 which is a distinct but serologically related virus (Fig. 1b).

Mice were primed by i.n. inoculation of live EHV-1, heat-inactivated EHV-1 or a mock-infected RK cell lysate. Live virus-inoculated mice developed clinical signs and some died, according to the inoculum dose. Mice inoculated with heat-inactivated virus or uninfected cell lysate showed no clinical signs and none died. Three weeks later, surviving donor mice were used to provide spleen cells for transfer to recipient mice. The number of cells transferred ranged from $8 \times 10^6$ to $4 \times 10^7$ among the different experiments. Recipient mice were challenged later on the day of i.n. transfusion with live EHV-1 and the clinical signs noted. Mice were killed on days 3 and 5 after transfer and the virus titres in lungs and turbinate bones were measured. In some cases DTH was measured 3 or 4 weeks later by means of a skin test and antibody levels in serum were also determined by means of an ELISA.

Priming with live virus produced a protective effect in recipient mice that received $4 \times 10^7$ spleen cells. Although there was no difference in virus titre on day 3, by day 5, there was a clear reduction in virus in both lungs and nasal turbinates (Fig. 2a). The reductions were significant ($P < 0.05$) and the degree of virus reduction correlated with the number of cells transferred. When the number of cells was reduced to $< 10^7$ the reduction in virus titre was not significant (data not shown). The reductions in virus titre were also reflected in reduced clinical signs in the inoculated mice. These reduced signs became particularly apparent beyond the 5th day p.i. Thus the transfer of spleen cells conferred protective immunity on the recipients. The transfer of spleen cells was more effective in reducing virus titres in lungs (at least $2 \log_{10}$) compared to turbinates (approximately $1 \log_{10}$). The spleen weights in recipients were found to correlate with protection. On day 5 p.i., the mice that received $4 \times 10^7$ immune spleen cells had the heaviest spleens ($128 \pm 11.2$ mg) followed by mice given $1 \times 10^7$ immune spleen
cells (107 ± 12.4 mg). The weight of spleens from mice that received spleen cells from mice primed with cell lysate was 82 ± 9.6 mg and the weight of normal spleens ranged from 80 to 100 mg.

In contrast to the results above spleen cells transferred from donors primed with heat-killed virus produced no reduction in virus titre in the lungs and turbinate of recipients following challenge (Fig. 2b). Furthermore, there appeared to be a significant increase in growth of virus in lungs and turbinate on day 5 p.i. These differences were even more apparent when a larger challenge dose was employed (4 × 10⁶ p.f.u.) causing all recipient mice to die compared with 25% mortality in unimmunized controls and 0% mortality in mice that received spleen cells from live virus-primed donors (data not shown). A dense mononuclear cellular infiltration with oedema was seen in histological sections of lungs obtained from these mice on day 5 p.i. Similar observations were made in mice that received spleen cells from donors primed with live EHV-1 but the cellular infiltration appeared to be less intense.

Serum antibody responses were measured in the groups of mice used above. The results (Fig. 3a) indicate that a low but measurable antibody response was recorded consistent with previous observations. When compared at particular times the antibody levels were not significantly different between the experimental groups. It was notable, however, that at five times (days 3, 7, 14, 21 and 28 p.i.) the antibody levels were lower in the mice receiving immune cells although different mice were sampled on each occasion.

Similar small reductions in antibody responses were observed in mice that received spleen cells from the donor mice primed with heat-inactivated EHV-1 (Fig. 3b). A lower challenge dose was employed (2 × 10⁴ p.f.u. compared with 5 × 10⁶ p.f.u. used previously) and the antibody titre obtained was generally lower. As before a reduced antibody response was observed (days 5, 14, 21 and 28 p.i.) although the differences between individual time points were not significant.

Surviving mice were also tested for DTH by innoculating a dose of heat-inactivated EHV-1 into the ear pinna and measuring the increase in skin thickness. The results, summarized in Table 1, show that recipient mice acquired an enhanced DTH response with maximal response obtained from live virus-primed donors and this DTH response correlated with the number of cells transferred. The data shown in Table 1 were obtained from the same group of mice used to measure virus titres in the organs (Fig. 2 and 3). A positive response was observed also in mice receiving spleen cells from donors primed with a single inoculation of heat-inactivated virus.

One of the important findings to emerge from this study was that a DTH response developed in mice inoculated with EHV-1, and the magnitude of response following i.n. inoculation was higher compared to that following i.v. inoculation. By contrast, i.v. inoculation gave a higher antibody response (M. Azmi & H. J. Field, unpublished). The enhanced CMI response following i.n. inoculation, despite the lower antibody response, may explain the observed protective immunity in upper and lower respiratory tracts against re-infection, suggesting that the elimination of EHV-1 from the site of replication is a function of CMI. It was intriguing that a DTH response was also shown to the EHV-4 antigen which is known to cross-react with EHV-1 serologically. We have recently observed both exacerbated responses to and protection against EHV-1 challenge following primary inoculation with EHV-4 in mice. This provides evidence for interaction between the two virus types with a fine balance between protective and deleterious cell-mediated responses (M. Azmi & H. J. Field, unpublished); this may be an important epidemiological factor in the natural host and is currently being investigated further.

Further evidence for the protective role of immune cells was obtained directly by means of adoptive transfer
Table 1. The effect of transferring spleen cells from primed donors on the increase of the ear thickness (DTH response) in recipients after i.n. inoculation with live EHV-1

<table>
<thead>
<tr>
<th>Source of spleen cells*</th>
<th>No. cells transferred</th>
<th>DTH response†</th>
<th>Virus protection‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (uninfected)§</td>
<td>0</td>
<td>25.93 aIJ</td>
<td>NA</td>
</tr>
<tr>
<td>RK cell lysate</td>
<td>8.3 x 10⁶</td>
<td>72.85</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>4 x 10⁷</td>
<td>73.07</td>
<td>—</td>
</tr>
<tr>
<td>Inoculated mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-inactivated virus</td>
<td>2 x 10⁷</td>
<td>107.41</td>
<td>—</td>
</tr>
<tr>
<td>Live virus</td>
<td>8.3 x 10⁶</td>
<td>112.33</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4 x 10⁷</td>
<td>131.30</td>
<td>+</td>
</tr>
</tbody>
</table>

* Donor mice were primed with the material shown by i.n. inoculation, 3 weeks before transfusion of spleen cells into recipients.
† Percentage ear thickness (mean from a group of four mice) increase at 24 h following antigen inoculation; test carried out 3 weeks after i.n. inoculation with EHV-1.
‡ +, Marked reduction (at least 1 log<sub>10</sub> p.f.u.) of virus titre in lungs with reduced clinical signs; —, no reduction of virus titre or clinical signs.
§ Naive mice tested for DTH response with viral antigen.
| NA, Not applicable.

studies. Transfer of immune spleen cells has been reported to protect against EHV-1 infection, e.g. a passive transfer of immune spleen cells has been shown to be protective in hamsters to EHV-1 infection (Sentsui et al., 1991). However, the route of EHV-1 infection in that model (intraperitoneal inoculation) and virus target organs (liver and spleen) are not those normally involved in the natural equine disease. Rapid clearance of herpes simplex virus from the ear pinna of mice was shown following adoptive transfer of immune cells (Nash et al., 1980, 1981). Results of the present adoptive cell-transfer experiments showed that the recipients were protected against challenge with EHV-1, if the mice were given spleen cells from mice primed with live EHV-1, although the elimination of infectious virus was found to be less effective in the upper compared to the lower respiratory tract. However, it appeared that the number of cells transferred is a critical factor in effecting protection since protection was not obtained in mice that received <10⁷ immune spleen cells.

The results of adoptive cell-transfer experiments in which donors were primed with heat-inactivated virus showed that the recipients were not protected against challenge with EHV-1; indeed virus replication was enhanced. An intense cellular infiltration in lungs may have resulted from antigen expression in the infected tissue focusing activated cells with consequent release of inflammatory mediators. This was consistent with the profound inflammatory exudate observed in the sections of lungs obtained from recipients. We also found that the cell number may be also a crucial factor in the enhancement of virus replication.

Transfer of cells from donors primed with live or heat-inactivated virus was found to enhance the DTH response compared to that seen following transfer from control donors. The magnitude of response was attributed to the number of spleen cells being transferred as well as to the type of antigen used for the stimulation of the donors. However, this did not correlate with protection from infection.

Serum antibody responses were obtained in recipients that received spleen cells from donors primed with live or heat-inactivated EHV-1. Compared to the control mice (which received spleen cells from mice primed with uninfected cell lysate) a slight suppression of serum antibody response was noted at several different time points although the difference between individual time-points was not significant. Lower antibody response in these cases may correspond to the induction of active cytotoxic CD8<sup>+</sup> lymphocytes in the population of transferred spleen cells, which could have a suppressor role reflected in the reduced antibody production.

Results described in this study demonstrate the potential of the murine model for the study of the immune response to EHV. Further experiments are in hand to define specific populations of immune cells involved in inducing the responses to EHV-1 and EHV-4 and the effector mechanism involved in virus clearance. These results suggest mechanisms by which these responses may be involved in EHV pathogenesis in the horse and how these responses may be used in the design of immunoprophylactic treatments.

We wish to thank Drs A. A. Nash, J. S. Gibson and A. R. Awan for their helpful discussion of data and Ms Alana Thackray for her expert technical assistance. M. Azmi was supported by a scholarship from the Universiti Pertanian Malaysia and a grant from the Jowett Trust, Cambridge University. We gratefully acknowledge substantial support for this work by the Equine Virology Research Foundation.

**References**


(Received 27 July 1992; Accepted 7 October 1992)