An investigation of the therapeutic value of vaccinia-immune IgG in a mouse pneumonia model

Mansun Law,† Mike M. Pütz and Geoffrey L. Smith

Department of Virology, Faculty of Medicine, Imperial College London, St Mary's Campus, Norfolk Place, London W2 1PG, UK

Vaccinia-immune globulin (VIG) was used to treat severe complications of smallpox vaccination, but its use was controversial because it resolved disease in only some clinical cases. VIG is a pool of hyperimmune sera collected from individuals with a high neutralizing titre against the intracellular mature form (IMV) of vaccinia virus (VACV), but activity against the extracellular enveloped form (EEV) was often not considered. Here, the efficacy of anti-VACV antibodies (Abs) in protecting mice from intranasal infection with the VACV strain Western Reserve (WR) was evaluated. Mice were immunized passively with hyperimmune rabbit Abs (IgG) generated against inactivated IMV or produced following infection by VACV; subsequently, animals were challenged with VACV WR. The results demonstrated that: (i) good protection requires Abs to EEV in addition to IMV; (ii) Abs were effective when given before or up to 4 days after infection; and (iii) protection of mice from VACV WR correlated with a reduction of virus replication in lungs, but not in brain. In agreement with studies conducted before smallpox was eradicated and recent studies using EEV antigens for immunization, this study reiterates the importance of anti-EEV Abs in protecting against orthopoxvirus infection and illustrates the need to evaluate both anti-IMV and anti-EEV neutralizing Abs in VIG.

INTRODUCTION

Orthopoxviruses are large DNA viruses that replicate in the cytoplasm and produce multiple forms of infectious virions (Moss, 2001; Smith et al., 2002). The genus Orthopoxvirus includes variola virus (VAR), the cause of smallpox; vaccinia virus (VACV), the vaccine against smallpox; cowpox virus (CPV); monkeypox virus (MPV); ectromelia virus (ECT); camelpox virus (CMPV); raccoonpox virus; and taterapox virus. These viruses are all antigenically related and infection with any one provides protection against each member of the genus (Fenner et al., 1989).

During virus replication, most infectious virus remains in the cytoplasm as intracellular mature virus (IMV) until cell lysis, but some IMV particles are wrapped by intracellular membranes to form intracellular enveloped virions (IEV). IEV migrate to the cell surface, where their outer membrane fuses with the plasma membrane to expose enveloped virions outside the cell. Particles retained on the cell surface are termed cell-associated enveloped virus (CEV) and those released are called extracellular enveloped virus (EEV). CEV and EEV are physically indistinguishable and mediate virus spread within the host, whereas IMV may spread infection between hosts. The morphogenesis of VACV has recently been reviewed (Smith et al., 2002; Smith & Law, 2004). Several EEV-specific proteins have been identified and two of these, A33R and B5R, are targets of protective antibody (Ab) responses (Galmiche et al., 1999; Hooper et al., 2000; Law & Smith, 2001; Bell et al., 2004; Fogg et al., 2004).

Despite being an effective smallpox vaccine, vaccination with live VACV caused complications such as fever, severe headaches, local pain, rash and erythema (Fenner et al., 1988; Thorne et al., 2003). Rarely, serious complications like eczema vaccinatum, generalized vaccinia and progressive vaccinia occurred that were life-threatening, particularly to immunocompromised individuals. Although safer VACV strains, such as Lister and New York City Board of Health (NYCBH or Wyeth), were used widely in the smallpox-eradication campaign, the complications of smallpox vaccination prompted the development of more attenuated vaccines, such as LC16m8 in Japan and modified vaccinia Ankara in Germany.

Inactivated virus vaccines were also tested. These contained predominantly IMV that was released by disruption of infected cells and inactivated by chemical or physical methods. Studies in humans (Kaplan, 1962; Kaplan et al., 1965) and animals (Madeley, 1968; Boulter et al., 1971; Turner & Squires, 1971; Appleyard & Andrews, 1974) demonstrated that killed vaccines were ineffective in
preventing orthopoxvirus infection, despite the presence of high levels of Abs to IMV (Kaplan et al., 1965; Boulter, 1969; Boulter et al., 1971; Turner & Squires, 1971). Notably, killed IMV vaccines did not induce Abs to EEV, did not prevent secondary plaque formation by virus growing in cell monolayers and did not neutralize virus (EEV) released into the supernatants of infected cells (Madeley, 1968; Boulter, 1969; Appleyard et al., 1971; Turner & Squires, 1971). Depletion of VACV-immune Abs with EEV antigens resulted in the reduction of the neutralizing activity against EEV (Appleyard et al., 1971; Bell et al., 2004).

Vaccinia-immune globulin (VIG) is recommended for the treatment of serious complications of smallpox vaccination, although its therapeutic value has not been fully proven (Kempe, 1960; Bray & Wright, 2003; Thorne et al., 2003; Hopkins & Lane, 2004) (http://www.bt.cdc.gov/training/smallpoxvaccine/reactions/vig.html). VIG is a pool of γ-globulin collected from patients who had recovered from or been vaccinated against smallpox (Kempe et al., 1956; Anderson & Skegg, 1970). VIG neutralized VACV in vitro; however, no distinction was made between anti-IMV and anti-EEV Abs and the assays used suggested that only Abs to IMV were measured and this was taken as an overall measure of virus-neutralization titre.

Here, the therapeutic potential of polyclonal IgG to IMV and EEV was evaluated by using a mouse intranasal-challenge model (Turner, 1967; Williamson et al., 1990). VACV-immune IgG (rabbit and human) was administered intraperitoneally at different times and animals were challenged by the neurovirulent VACV strain Western Reserve (WR) (Turner, 1967; Payne, 1980; Williamson et al., 1990). VACV WR was derived from VACV strain NYCBH by multiple passages in mouse brain (Fenner et al., 1988). Some early studies of protection against orthopoxviruses used the rabbit–rabbithpox virus model (Bedson & Duckworth, 1963) and measured fever and mortality, but other parameters and the recovery phase after infection were not reported (Madeley, 1968; Boulter, 1969; Appleyard et al., 1971; Boulter et al., 1971; Turner & Squires, 1971; Boulter & Appleyard, 1973; Appleyard & Andrews, 1974). In this study, we measured the outcome of infection by changes in body weight and the development of signs of illness (SOI) during both the onset and recovery phases of infection.

The results showed that protection was better if Abs to both IMV and EEV were present, and disease could be ameliorated if Abs were administered either before or up to 4 days post-infection (p.i.). Weight loss and SOI correlated with virus replication in the lungs, but not brain, as thought previously. This study emphasizes the importance of evaluating anti-EEV activity in VIG.

METHODS

Antibody. Hyperimmune rabbit Abs raised following infection with live VACV strain WR or International Health Department (IHD)-

and after immunization with inactivated WR IMV were described previously (Law & Smith, 2001). IgG of the final bleeds was purified by using Protein A–Sepharose columns (Amersham Biosciences). Human vaccinia-immune IgG (huVIgG) was prepared by using a Protein G–Sepharose column (Amersham Biosciences) from a 48-year-old male who had been vaccinated five times, including a booster with VACV strain Lister 5 weeks before the serum was prepared. Two other sera from humans immunized multiple times against smallpox and a sample of VIG (originally from CDC, Atlanta, GA, USA, and obtained from T. Monath, Acambis) were also tested. Abs were dialysed against PBS and quantified by spectrophotometry.

Virus titration and neutralization assay. VACV strain WR was used. Purified IMV and fresh EEV were produced from RK13 and BHK-21 cells, respectively, as described previously (Law & Smith, 2004). The IMV- and EEV-neutralizing titres of IgG samples were determined by plaque-reduction assay on RK13 cells (Law & Smith, 2001).

ELISA. Soluble forms of recombinant A33R, A56R and B5R proteins fused to the biotin acceptor peptide (BAP) (O’Callaghan et al., 1999) were produced in chinese hamster ovary (CHO) cells and purified by affinity chromatography using an anti-BAP monoclonal antibody (mAb). VACV WR antigens were produced in RK13 cells and UV-inactivated (Hanson et al., 1978). IgG titres were determined by using a protocol modified from that of Putz et al. (2004). Briefly, 96-well plates were coated overnight with 100 ng purified BSR, A33R, A56R or BSA per well (negative control; Sigma-Aldrich) or VACV cell lysate corresponding to 100 μl in 50 μl carbonate/bicarbonate buffer per well. Plates were washed and blocked for 120 min with 200 μl 5 % dextrin skimmed milk in Tris/acetate buffer. After washing, plates were incubated for 90 min with 50 μl threefold serial Ab dilutions in Tris/acetate buffer containing 1-0 % milk and 0-1 % Tween 20. Rinsed plates were incubated with 50 μl goat anti-rabbit or anti-human (γ-chain-specific) IgG–alkaline peroxidase conjugate (diluted 1:1000; Sigma-Aldrich) for 60 min. Finally 100 μl p-nitrophenyl phosphate substrate (SIGMA FAST; Sigma-Aldrich) was added and the OD450 was recorded after 60 min (Multiskan Ascent; Thermo Electron Corporation). Linear-regression plots were determined for each Ab sample and end-point titres were defined as the reciprocal of dilutions corresponding to twice the mean OD450 values obtained with BSA.

Virus-challenge experiments. VACV WR was prepared from infected RK13 cells by sedimentation through a 36 % (w/v) sucrose cushion and virus infectivity was titrated on BS-C-1 cells (Law & Smith, 2004). The titre of virus administered to animals was redetermined in each experiment. Six- to eight-week-old female BALB/c mice (16–22 g) were immunized passively with IgG (in 200 μl) by intraperitoneal injection before or after virus challenge. The half-lives of rabbit and human IgG in mice following intravenous injection were approximately 5-6 and 8 days, respectively (Gitlin et al., 1976). For virus challenge, mice were anaesthetized with isoflurane and inoculated intranasally with 20 μl (10 μl each nostril) of virus. Body-weight change and SOI of mice were recorded daily and mice that reached humane end points (loss of 30 % body weight) were euthanized. SOI were recorded as described previously (Alcami & Smith, 1992). Scores: 0, no SOI; 1, slight ruffling of fur; 2, slight back arching, more ruffling, slightly reduced mobility; 3, pneumonia, slight shaking, increased back arching, increased ruffling, clearly reduced mobility; 4, pneumonia, more shaking, huddled appearance, extensive ruffling, very little mobility. To measure virus in organs, mice were sacrificed and the blood, lungs, spleens and brains were collected. The tissues were stored frozen in culture medium. Thawed tissues were homogenized by using a syringe plunger and filtered through a 70 μm cell strainer. The homogenates were freeze-thawed three times to release virus, which was titrated on RK13 cells.
**Statistical analysis.** Correlation between the log_{10}-transformed rabbit IgG ELISA titres and IMV- or EEV-specific 50% plaque reduction (ND_{50}) values was analysed by a two-tailed Pearson correlation test (GraphPad Prism software, version 3.0). The Pearson product-moment correlation coefficient r is indicated where relevant. Comparisons between the mean percentage body-weight change and SOI for different groups were performed by using an unpaired, two-tailed Student’s t-test (Microsoft Excel software, version 2002). P<0.05 was considered significant.

**RESULTS**

**Neutralizing titre of antibody**

Seven Abs (six rabbit and one human) were used in this study. Some of the rabbit Abs were described previously (Law & Smith, 2001). Rb-WR1, Rb-WR2, Rb-IHDJ1 and Rb-IHDJ2 were generated by intradermal infection (four times in 3 months) of New Zealand White rabbits with live WR of IHD-J IMV, whereas Rb-deadWR1 and Rb-deadWR2 were raised by inoculation of rabbits using non-infectious WR IMV particles. The human Ab (huVIgG) was obtained from a hyperimmune male. Neutralizing activities of the IgGs against IMV and EEV were determined by plaque-reduction assay and are expressed as the IgG concentration required to achieve 50% plaque reduction (ND_{50}) (Table 1). The rabbit and human Abs produced following live infection had similar anti-IMV activity (within twofold of that of Rb-WR1). In contrast, Rb-IHDJ1 had only 33% anti-EEV activity compared to Rb-WR1, and huVIgG had only 3% that of Rb-WR1. The two Rb-deadWR Abs were about 4-5-fold more potent in neutralizing IMV than Rb-WR1. Unexpectedly, Rb-deadWR1 also had a comparable anti-EEV activity (74%) to Rb-WR1, whereas that of Rb-deadWR2 was only 16% of that of Rb-WR1. This anti-EEV activity may reflect residual EEV antigens present in the IMV preparation or limited gene expression in infected cells, because no infectivity was detected in the inactivated IMV by plaque assay.

**Table 1. Neutralizing titre of antibody (ND_{50})**

The titres (ND_{50}) shown are the amount of IgG required to neutralize 50% of IMV or EEV in a plaque-reduction assay using RK13 cells.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Anti-IMV</th>
<th></th>
<th>Anti-EEV</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Titre (µg ml(^{-1}))</td>
<td>Percentage of Rb-WR1</td>
<td>Titre (µg ml(^{-1}))</td>
<td>Percentage of Rb-WR1</td>
</tr>
<tr>
<td>Rb-WR1</td>
<td>1.02</td>
<td>100</td>
<td>4.0</td>
<td>100</td>
</tr>
<tr>
<td>Rb-WR2</td>
<td>1.35</td>
<td>76</td>
<td>3.7</td>
<td>108</td>
</tr>
<tr>
<td>Rb-IHDJ1</td>
<td>1.48</td>
<td>69</td>
<td>12.1</td>
<td>33</td>
</tr>
<tr>
<td>Rb-IHDJ2</td>
<td>1.03</td>
<td>99</td>
<td>3.7</td>
<td>108</td>
</tr>
<tr>
<td>Rb-deadWR1</td>
<td>0.22</td>
<td>464</td>
<td>5.4</td>
<td>74</td>
</tr>
<tr>
<td>Rb-deadWR2</td>
<td>0.24</td>
<td>425</td>
<td>24.4</td>
<td>16</td>
</tr>
<tr>
<td>huVIgG</td>
<td>1.96</td>
<td>52</td>
<td>15.0</td>
<td>4</td>
</tr>
</tbody>
</table>

**ELISA**

Specificity of the rabbit (Fig. 1a) or human (Fig. 1b) Abs to IMV and EEV antigens was investigated by ELISA. IMV antigens are abundant in VACV-infected cells and were used in ELISA after diluting the UV-inactivated, infected-cell lysate 8000-fold. At this dilution, the EEV antigen A33R was not detected by using A33R-specific mAbs and only very low levels of B5R and A56R antigens were found by using mAbs to A56R or B5R (data not shown). The anti-EEV activity of the IgGs was evaluated by using recombinant soluble A33R, A56R and B5R proteins produced from CHO cells.

Despite Rb-deadWR1 and Rb-deadWR2 having greater than fourfold higher IMV-neutralizing activity than other rabbit IgGs, these Abs did not have a higher activity against IMV than Rb-WR1 or Rb-WR2 when measured by ELISA. This suggests that immunization with live and killed virus may result in qualitatively different Abs.

The IMV-specific ND_{50} and VACV-specific ELISA titres did not correlate exactly (Pearson r = −0.59, P = 0.22); however, there was a significant correlation between EEV-neutralization titre and the level of B5R-specific IgG (Pearson r = −0.94, P < 0.01). Rb-WR1, Rb-WR2, Rb-IHDJ2 and Rb-deadWR1 had an end-point ELISA titre...
between 220 000 and 320 000 and neutralized 50 % of EEV (ND50) at <6 µg ml⁻¹. The less potent Rb-IHDJ1 and Rb-deadWR2 had anti-B5R ELISA titres of 62 000–68 000 and their ND50 titres were 12 and 24 µg ml⁻¹, respectively. No correlation was observed between anti-A33R (P=0.09) or anti-A56R (P=0.09) ELISA titres and EEV neutralization. Although Rb-IHDJ1, Rb-deadWR1 and Rb-deadWR2 contained similar anti-A33R and anti-A56R activity, their EEV-neutralizing titres varied considerably. These data agree with a recent report that B5R is the major determinant for EEV neutralizing titres varied considerably. These data agree with a recent report that B5R is the major determinant for EEV neutralizing titres.

Although the Abs produced from live infection in rabbits and humans all had a higher anti-IMV ND50 than anti-EEV, the difference was greatest in huVIgG (Table 1). In Fig. 1(b), the ELISA titres to the EEV and VACV antigens of huVIgG were compared with several other human samples. huVIgG was derived from serum 1 and showed the same ratio of ELISA titres to both IMV and EEV antigens. This ratio varied in other samples and, notably, anti-A56R reactivity was higher in huVIgG (serum 1) and the commercial VIG than anti-B5R and -A33R reactivity, whereas anti-B5R reactivity was higher in sera 2 and 3. Serum 1 (huVIgG) had ELISA titres equivalent to, if not better than, the other human samples and it was therefore used as a representative human sample for in vivo study (see below).

Therapeutic activity of Abs in vivo

Respiratory infection of mice has been used to investigate VACV virulence and simulates the natural route of infection by VAR (Williamson et al., 1990; Tscharke et al., 2002; Schriewer et al., 2004). Different VACV strains have differing virulence in this model and the more virulent strains, such as WR and IHD-J, have been used most often. To evaluate the potency of Abs in treating poxvirus infection, IgG was transferred passively by intraperitoneal injection before mice were infected intranasally with VACV WR.

The optimal dose of IgG and challenge virus was determined in pilot studies using small groups of animals (n=3) (data not shown). A range of Rb-IHDJ1 IgG (15, 40, 100 and 250 µg) was injected intraperitoneally 1 day before challenge with 10⁴ p.f.u. VACV WR. This virus dose causes the mice to lose 25–30 % of their body weight. Administration of IgG reduced weight loss and SOI in a dose-dependent manner although, even at 250 µg, there was some weight loss (14 versus 23 % of control rabbit IgG on day 7 p.i.) and SOI (score of 1.5 versus 3 for the control). The lowest IgG dose (15 µg) reduced SOI (to a score of 2) and weight loss, so that the humane end point was not reached by day 8 (whereas one mouse in the control group was euthanized). At a lower dose of challenge virus (10³ p.f.u.), 250 µg Rb-IHDJ1 prevented all weight loss and SOI were reduced to a score of 0.5 (control IgG, 15 % weight loss and an SOI score of 2.5). A slightly higher challenge dose (3 × 10³ p.f.u.) after injection of 250 µg Rb-IHDJ1 caused 5 % weight loss and an SOI score of 1 on day 7 (control IgG, 15 % weight loss and an SOI score of 2.5). Increasing the dose of Rb-IHDJ1 to 500 µg per mouse (3 × 10⁴ p.f.u. challenge) did not improve protection further. So, under these conditions, passive administration of Ab did not provide complete protection.

High-dose virus challenge: 5 × 10⁴ p.f.u.

The ability of passive Ab to protect mice from reaching the humane end point was tested by using a high-dose virus challenge (Fig. 2). Mice (n=5) were injected with 500 µg of each of the rabbit IgGs listed in Table 1. Additionally, the concentrations of Rb-deadWR1 and Rb-deadWR2 IgG were adjusted to contain an anti-IMV activity (termed Rb-deadWR/IMV) equivalent to that present in Rb-IHDJ1. All mice were challenged with 5 × 10⁴ p.f.u. 1 day after Ab transfer. As found in pilot experiments, this dose of Ab did not prevent weight loss following challenge. During the 6 days after challenge, all groups, except Rb-deadWR1 (500 µg) and both Rb-deadWR/IMV IgG, lost between 15 and 18 % weight, whilst the control groups and the exceptions lost 22–26 %. However, all Rb-deadWR groups developed higher SOI by day 6 p.i. (score 2–3) than groups that were given IgG derived from live infection. On day 7, some animals started to recover. Compared with group Rb-WR1 (lowest weight loss and SOI), control groups, Rb-IHDJ1, Rb-deadWR1 and both Rb-deadWR/IMV lost significantly more weight (P<0.05), and control groups, both Rb-deadWR and Rb-deadWR/IMV developed greater SOI (P<0.05). Notably, when the anti-IMV titre of Rb-deadWR IgG was normalized to that of Rb-IHDJ1, the SOI increased (days 5–7) and some animals in these groups reached humane end points. These data demonstrated that, by using a high virus challenge: (i) passive vaccinia-immune IgG was unable to prevent weight loss and SOI, but could protect mice from reaching the humane end point; and (ii) the best protection observed required Abs to EEV as well as IMV.

Low-dose virus challenge: 3 × 10³ p.f.u.

The ability of Ab administered passively to protect mice from disease was tested by using a low-dose virus challenge (Fig. 3). Mice (n=8) were injected with 500 µg IgG of Rb-WR1, Rb-IHDJ2, Rb-deadWR1, Rb-deadWR2, Rb-deadWR1/IMV or Rb-deadWR2/IMV and then challenged with 3 × 10³ p.f.u. 1 day later. Mice in the control groups lost 16–18 % weight and developed an SOI score of 3 by day 7 p.i. As seen with the high-dose virus challenge, the Abs with higher anti-EEV activity (Rb-WR1, Rb-IHDJ2 and Rb-deadWR1) provided better protection, reducing both weight loss and SOI. Despite having higher or equivalent levels of anti-IMV activity, Rb-deadWR2 (weight loss 6 %, SOI score of 1) and the other two antibodies with normalized anti-IMV activity (weight loss 12–16 %, SOI score 1:5–2) provided inferior protection. From days 6 to 10, these groups had significantly different weight loss and SOI compared to Rb-WR1 (P<0.05, except on days 6 and 9, where SOI of Rb-deadWR2 had P<0.08). This again
demonstrated an important role of anti-EEV Abs in immunoprophylaxis.

A lower-dose virus challenge also allowed recovery from disease (after day 7 p.i.) to be studied. Mice in control groups only recovered to 14% weight loss and an SOI score of 2 by day 12 p.i., but mice receiving Abs derived from a live infection recovered almost completely by day 9 p.i. Mice receiving Abs with low anti-EEV activity recovered better than controls, although more slowly than groups receiving Abs derived from a live infection.

### huVIgG versus rabbit VIG

The mouse intranasal-infection model is a useful model to test the therapeutic potential of VIG before clinical trials. Here, a human sample, huVIgG (500 μg), was compared to the rabbit Abs (Fig. 4). After infection with $10^5$ p.f.u., mice that received huVIgG had slightly reduced weight loss.
and SOI compared to mice receiving non-immune rabbit IgG (days 5–10, weight change, \( P > 0.15 \); SOI, \( P > 0.26 \)). Rb-deadWR2/IMV lost weight and developed SOI like the control group, but recovered more quickly (weight change on days 7–10 and SOI on days 8–10, \( P < 0.04 \)). However, at a dose of \( 10^3 \) p.f.u., mice given huVlgG developed significantly less SOI (\( P < 0.05 \), days 7–10) than those given control human IgG and lost less weight, but this was statistically insignificant (\( P > 0.15 \)). At both doses of challenge virus, the groups that received Rb-WR1, Rb-deadWR1 or Rb-deadWR2/IMV were better protected. Because the half-life of rabbit IgG in mice is not longer than that of human IgG, these results were consistent with the observation that huVlgG contains much lower anti-EEV activity than the other two Abs (Table 1).

Collectively, these data show that the in vitro virus-neutralization titre against IMV and EEV can be used to predict the efficacy of Abs in protecting mice from respiratory infection by VACV, and that Abs to EEV are important in protecting against poxvirus infection.

**Time of antibody administration**

VIG is used for the prevention and treatment of vaccine-related complications and so the effect of time of administration on the efficacy of the treatment was investigated.
Mice were immunized passively with Abs 1 day before or 2, 4 or 6 days after challenging with 3 × 10³ p.f.u. This virus dose was selected to ensure that all animals did not reach the humane end point and so the recovery of all animals, including those receiving control IgG, could be monitored. Animals receiving control IgG started to lose weight from day 5 p.i., reached maximum weight loss (19%) by day 7 p.i. and recovered thereafter. SOI lagged 1 day behind weight loss, but showed a similar pattern and reached a maximum score (2–8) by day 8 p.i. Mice given Rb-WR1 (containing Abs to IMV and EEV) showed very significant improvement in both weight change and SOI (days 5–12 p.i., P < 0.04) if the IgG was given 1 day before or 2 days after infection. Similarly, if the Ab was delayed until day 4 (the day before untreated animals developed disease), there was still improvement in SOI (days 6–12 p.i., P < 0.04) and weight change (days 5–12 p.i., P > 0.09). However, passive transfer of Rb-WR1 on day 6 p.i. did not reduce weight loss, although the animals had a lower SOI score than the control group from days 6 to 9 p.i. (SOI days 6 and 8 p.i., P < 0.03). These data demonstrated that passive Ab is most beneficial when given before the onset of disease (day 4 p.i.) and its benefit decreased thereafter. Although there was a difference between the control group and mice receiving Rb-WR1 on day 6 p.i. in SOI, the treatment did not significantly reduce weight loss at the peak of disease (day 7 p.i.), nor did it aid the recovery of the animals, as measured by weight change and SOI. Similar data, albeit with a slightly lower level of protection, were seen after treatment with Rb-deadWR2/IMV (data not shown).

**Control of virus dissemination**

VACV WR was proposed to be neurovirulent because it spreads to and replicates in the brain following intranasal inoculation. If so, Abs able to restrict infection in the lungs may prevent virus spreading from the respiratory tract to the brain. Therefore, the spread of virus from the primary site of replication (lungs) to secondary sites (blood, spleen and brain) was monitored at different times after infection with 3 × 10³ p.f.u. in the presence or absence of different IgGs (Fig. 6). The outcome of infection (weight loss and SOI) was the same as seen previously with this dose of challenge virus (See Supplementary Table in JGV Online). Viraemia was seen only on day 5 p.i. in mice given control IgG and not in mice given anti-VACV Abs. At all times p.i., in primary (lung) and secondary (spleen) sites of infection, there was an inverse relationship between Ab titres to VACV (Abs to IMV and EEV) and virus titres, suggesting that Abs can suppress virus replication and spread. In contrast, virus titres in the brain did not follow the same pattern. On days 2 and 5 p.i., the presence of anti-VACV Abs had decreased the virus titre in brain. However, on day 8 p.i., during the recovery phase of infection, neither anti-VACV Abs reduced virus titres in the brain. Similar results were obtained in an independent experiment with mice given 250 μg Rb-IHDJ1 IgG and challenged with 10³ or 10⁴ p.f.u. VACV WR (see Supplementary Figure in JGV Online). These results showed that: (i) there was no correlation between the level of virus in the brain and the weight loss, SOI or behaviour of the animals; and (ii) mice that received anti-VACV IgG recovered better than those given control IgG, despite the levels of virus in the brain being indistinguishable between these groups.

These data suggest that the disease in mice infected by VACV WR is a function of virus replication in the lungs and pneumonia, rather than the neurovirulence of the virus, as thought previously.

**DISCUSSION**

This study revisited the ability of passive Ab to protect against orthopoxvirus infection and, unlike some other studies, investigated the effects of Abs to both IMV and EEV. Heterologous polyclonal Abs were used because there

---

**Fig. 5.** Time of Ab administration. Rb-WR1 IgG (500 μg) was injected intraperitoneally into mice (n = 6) on days −1, 2, 4 or 6 and the mice were challenged with 3 × 10³ p.f.u. VACV WR virus on day 0. The mean ± SEM daily body-weight change (%) and SOI of each group are shown.
was no EEV-neutralizing mAb available and the mouse intranasal model was selected so that rabbit Abs and human VIG could be studied.

The IMV and EEV ND50 and ELISA titres of each IgG were determined, allowing a systematic comparison of the samples by in vitro assays before animal testing. The values of Rb-IHDJ1 and Rb-WR2 are similar to those obtained in our previous study (Law & Smith, 2001); the slight differences may be due to different batches of purified IgG and the use of a new protocol to produce higher levels of intact EEV (Law & Smith, 2004). It was notable that both Rb-deadWR Abs have a higher anti-IMV activity (about 4-5-fold) than IgG derived from repeated live infection (Rb-WR1). Similar observations were made previously (Boulter et al., 1971; Appleyard & Andrews, 1974) and this may be due to the enhancing effect of the adjuvant used for immunization and/or the presence of virus-encoded immunomodulatory proteins during a live infection. Another notable feature was the relatively low anti-EEV titre of huVIG, despite multiple immunizations.

Studying the specificity of the Ab samples by both ELISA and virus-neutralization test was informative and revealed that immunization with live or killed IMV may result in qualitatively different Abs to IMV. Rb-deadWR1 and Rb-deadWR2 had greater than fourfold higher IMV-neutralizing titres than Rb-WR1 and Rb-WR2, but reacted to VACV-antigens (mostly IMV) in ELISA at a similar level. On the other hand, the EEV-neutralizing activity was directly proportional to the concentration of the anti-B5R Ab, but not anti-A33R or anti-A56R Ab. B5R is a target for both in vitro neutralization of EEV and in vivo protection (Galmiche et al., 1999; Law & Smith, 2001; Hooper et al., 2003, 2004; Bell et al., 2004). Abs to A33R do not neutralize EEV in vitro, although passive transfer of homologous, but not heterologous, Ab conferred some protection against virus challenge (Galmiche et al., 1999; Hooper et al., 2000).

The role of anti-EEV Ab in VIG in protecting against orthopoxvirus infection and the relationship between in vitro assays and in vivo protection were investigated. By using a high-dose virus challenge, although all mice groups lost weight and developed SOI early after disease onset, groups that received Abs with high anti-IMV and anti-EEV activity were protected from reaching the humane end point, whereas groups that received an equivalent level of anti-IMV, but lower anti-EEV activity, did appreciably worse and several animals were euthanized. Using a low-dose virus challenge, Abs with high anti-IMV and anti-EEV activity prevented nearly all disease, whereas weight loss and SOI were evident in groups that received an equivalent level of anti-IMV, but lower anti-EEV, activity. Protection against orthopoxvirus was dependent on the dose of passive Ab and the level of challenge virus.

Systemic orthopoxvirus infection was proposed to induce primary and secondary viraemia (Fenner et al., 1988). Virus entered and replicated in the lymphoid tissue of the respiratory tract, leading to a transient viraemia, infection of the reticuloendothelial system and further virus replication. After the asymptomatic incubation period (12 days for smallpox), a second viraemia containing a higher level of virus was accompanied by fever, multiple skin lesions and an acute immunological response. Passive Ab was suggested to suppress the secondary viraemia and, consequently, ameliorate the disease (Kempe et al., 1956).
Following the same principle, passive Ab in the mouse model would only be effective when administered before the onset of disease.

In this study, animals developed noticeable disease between 4 and 5 days p.i., depending on the virus dose. With a high virus dose, animals started to reach the humane end point by day 7 p.i., but with a lower dose (3 x 10^3 p.f.u.), the onset of disease and peak weight loss were delayed slightly (Figs 3 and 5). Passive Ab given on day 2 p.i. conferred protection almost as effectively as Ab given 1 day before infection, and Ab given on day 4 p.i. still provided significant benefit. However, administration of Ab on day 6 p.i. did not reduce weight loss, gave only a slight amelioration in SOI and did not aid recovery from infection (Fig. 5). Vaccination with VACV up to 4 days post-exposure to VAR ameliorated the severity of smallpox, but thereafter, vaccination conferred no benefit (Fenner et al., 1988). If data from the model used here can be extrapolated to human disease, it might suggest that treatment of smallpox or life-threatening vaccine-induced complications with VIG would be most effective either before disease onset or immediately thereafter. Delay beyond this might render VIG ineffective and other therapeutics would be needed.

The mouse intranasal-challenge model has been used to study the virulence of VACV strains and mutants (Turner, 1967; Williamson et al., 1990; Tscharke et al., 2002) and evaluate VACV immunogens (Galmiche et al., 1999) and smallpox vaccines (Belyakov et al., 2003; Wyatt et al., 2004). Other challenge routes, such as intraperitoneal injection, were also used, although the intraperitoneal route is unnatural and a much higher virus dose is required to cause disease (Hooper et al., 2000, 2003; Ramírez et al., 2002). For vaccine and Ab studies, VACV WR is often used because it is more virulent than many other VACV strains. The virulence of WR was thought to be associated with the neurotropism of the virus. However, data presented here showed that virus titres in the brain did not correlate with disease or recovery from infection. Instead, disease correlated with virus titres in the lungs, and the primary role of passive Ab was to suppress virus replication in lungs and prevent pneumonia.

Lastly, the in vitro assays and animal model described here are useful for the evaluation of heterologous antibody. huVIGG contains slightly lower anti-IMV and much lower anti-EEV activity in both plaque-reduction assay and ELISA than the rabbit hyperimmune IgG. Consistent with this, it is also less potent in vivo (Fig. 4). This emphasizes the need to assess the anti-IMV and anti-EEV activity of VIG separately and standardize the Ab according to both parameters. In a recent study of commercial VIG, this distinction was again neglected (Hopkins et al., 2004). Similarly, it is also important to measure the anti-IMV and anti-EEV neutralizing titres induced by smallpox vaccines, although EEV neutralization was not reported in recent studies (Earl et al., 2004; Hooper et al., 2004).

ACKNOWLEDGEMENTS

We thank P. Anton van der Merwe for advice about the production of soluble EEV proteins. This work was supported by grants from the Wellcome Trust and the UK Department of Heath. G.L.S is a Wellcome Trust Principal Research Fellow.

REFERENCES


