A model for vaccinia virus pathogenesis and immunity based on intradermal injection of mouse ear pinnae

David C. Tscharke and Geoffrey L. Smith

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Vaccinia virus (VV) proteins that interfere with the host response to infection are of interest because they provide insight into virus–host relationships and may affect the safety and immunogenicity of recombinant VV (rVV) vaccines. Such vaccines need assessment in animal models and with this aim a model of VV infection based on intradermal injection of BALB/c ear pinnae was developed and characterized. In this model, the outcome of infection is affected by the dose of virus inoculated but virus spread is minimal and the mice suffer no signs of systemic illness. Cellular and humoral immune responses to these infections were measured readily and were independent of virus dose over a 100-fold range. Thus the model seems suitable for the analysis of the safety and immunogenicity of VV mutants lacking specific immunomodulatory proteins or bearing foreign antigens.

All vaccinia virus (VV) strains tested encode proteins predicted to interfere with the host response to infection (Smith et al., 1997). Highly attenuated VVs such as NYVAC (Tartaglia et al., 1992) and modified vaccinia Ankara (MVA) (Antoine et al., 1998) that are candidate recombinant vaccines are no exception, although in MVA some of these proteins are absent (Blanchard et al., 1998). These proteins are of interest for virus pathogenesis and vaccine development. In each case it is important to understand the function of such proteins in vivo and this requires appropriate animal models. All models of VV infection may be criticized because the natural host(s) is unknown. However, experimentally the virus has a wide host range and, with a few notable exceptions, many of its immunomodulators are active and therefore can be studied across this spectrum of species (Smith et al., 1997).

The mouse is particularly amenable to studies of pathogenesis and immunity because of the availability of well-defined inbred strains, including a growing repertoire of targeted gene knockouts, and a wide range of immunological reagents. Mice can be infected with VV by many routes including intravenous, intraperitoneal, subcutaneous, footpad, intradermal, intracranial and intranasal (Nelson, 1938; Briody, 1959; Turner, 1967; Buller, 1985; Andrew et al., 1989). Intracranial and intranasal inoculation with moderate doses of VV strain Western Reserve (WR) cause severe infections in mice (Turner, 1967), allowing the relative virulence of virus mutants to be studied. However, the effects of some virus proteins may be too subtle to be detected in the context of these severe infections. Furthermore, severe infections are poor models for vaccination where clinical impact must be minimized. In contrast, mice infected with VV by cutaneous inoculation undergo a limited infection that is well tolerated even at high doses (Briody, 1959). The outcome of infection with VV mutants by other routes is not always a good predictor of their growth characteristics in skin (Lee et al., 1992), indicating that virus proteins may exert different effects at different sites of VV infection. Therefore, it seems prudent to investigate the function of VV proteins in a variety of in vivo situations. For these reasons a well-defined cutaneous model of VV infection would be a useful tool for studying the function of VV proteins.

A good model of VV infection should allow many parameters of infection to be assayed including clinical signs, growth of virus, infiltration of lesions and cellular and humoral immune responses. Possible sites for cutaneous inoculation not requiring shaving include the tail, footpad and ear. Tail scarification has been used in many studies but it is difficult to assay virus or to cut histological sections from this site. Infection of the ear pinna causes less distress than footpad inoculation, has been used previously as a model for other virus infections (Hill et al., 1975; Moorhead et al., 1999), and enables straightforward virological and histological analyses. VV has been used to infect mouse ears, but hitherto only virus titres were reported (Ikeda et al., 1991).

VV strain WR was grown and titrated in RK13 and BS-C-1 cells, respectively. In all experiments, 10 µl containing 10^4, 10^5 or 10^6 p.f.u. of VV was injected intradermally into the left ear pinnae of female, adult, BALB/c mice (minimum 8 weeks old) using a 27 gauge needle and a 100 µl Hamilton syringe.
Irrespective of dose, virus titre increased to a peak of approximately 10^6 p.f.u. per ear at 4 days p.i. and thereafter declined slowly until 10 to 12 days p.i. after which virus was mostly cleared from ears infected with 10^4 p.f.u.. The amount of virus replication that can occur in an infected ear appears to be limited because all doses gave the same peak titre. This reproducible effect was also reported for infected flank skin (Lee et al., 1992) and is probably due to innate defence mechanisms halting the lateral spread of virus. Different inoculation doses resulted in different virus titres at 2 days p.i. and inoculation with 10^4 p.f.u. resulted in a more rapid clearance that was apparent 12 days p.i. After 12 days p.i. with the higher doses, virus persisted in scabs (data not shown). Results from virus titrations were difficult to analyse in these cases as scabs, sometimes containing in excess of 10^5 p.f.u., fell off sporadically over a period of a week, causing sudden reductions in virus titres. No virus was detected in any mouse by 20 days p.i. The similar maximum titre obtained and the more rapid clearance of virus indicated that low doses of virus (10^4 p.f.u.) may be better for analysing differences amongst VV mutants.

Spread of virus from infected ears was examined by screening homogenates of left cervical draining lymph nodes (DLN), spleen, ovaries and brains for infectious virus. Tissues were taken from each of two mice given 10^4, 10^5 and 10^6 p.f.u. on 4, 8 and 12 days p.i. and the limit of detection was 5 p.f.u. per sample. With the exception of DLN on day 4, no virus was detected outside the ear at any time. The extent of the DLN infection was examined by screening nodes at 2, 4, 6, 9 and 12 days p.i. using the same three doses and three mice per dose on each day. Virus was detectable in DLN of all mice inoculated with 10^5 and 10^6 p.f.u. at 2 and 4 days p.i. In contrast, no mice infected with 10^4 p.f.u. had detectable virus in their DLN on day 2 and only two of three and one of three had virus on days 4 and 6, respectively. All virus titres in DLN were low, the highest being 160 p.f.u. and the mean of all positive results was 20 p.f.u.. These data demonstrate that in this model, doses of up to 10^6 p.f.u. of VV give an infection that is highly localized.

A histological examination of frozen sections of ears that had been infected with 10^6 p.f.u. was undertaken at different days p.i. (Fig. 2). A striking feature of infections was the thickening of the ear, best seen in row b. On closer examination (row c) this thickening had two causes: firstly, there was oedema and massive infiltration of the dermis containing CD4^+ and CD8^+ T lymphocytes, B lymphocytes, macrophages and other leukocytes (not shown); secondly, the epidermis itself was thickened, in some places up to 10 times the normal width, and this was probably due partly to expression of the VV growth factor (Buller et al., 1988). Measurement of the thickness of ears may provide another marker of the progress of infection and the level of infiltration, but at later times of infection the formation of scabs might distort the accuracy of these measurements. A monoclonal antibody against VV protein B5R (a component of the outer membrane of extracellular enveloped virus) was used to detect infected cells
Mouse ear model of vaccinia infection

Fig. 2. Histological analysis of ear pinna infection on various days after injection with 10^6 p.f.u. of VV. Appearance of ears before removal (row a), haematoxylin and eosin-stained sections at low power (row b) and high power (row c) and sections stained immunohistochemically with anti-VV B5R (row d) are shown. The day after inoculation is indicated at the top and the scales shown in the first column apply across each row.

(row d). On day 4 B5R antigen was confined largely to the epidermis but later most of the antigen was seen in round eosinophilic bodies in the middle of the ear. By 12 days p.i. B5R antigen was very scarce and was absent from 20 days p.i.

To analyse the host response to infection, VV-specific CD4⁺ and CD8⁺ T cells and antibody responses were measured. Cellular immunity to VV was assessed by the measurement of VV-specific, interferon-γ (IFN-γ)-secreting cells using a new ELISPOT technique. In published ELISPOT methods (Miyahira et al., 1995), T cells are stimulated by addition of peptide; however, as yet no T cell epitopes have been defined for VV. Nonetheless, we found that either UV/trioxsalen-inactivated intracellular mature vaccinia virus (IMV) (Hanson et al., 1978), equivalent originally to 10⁶ to 10⁷ p.f.u. per well, or P815 cells 4 to 8 h p.i. with VV (10 p.f.u. per cell) and γ-irradiated (3000 rads) at 10³ cells per well, were able to stimulate IFN-γ production by splenocytes from VV-infected but not uninfected mice. Further, by using monoclonal antibodies and complement to deplete splenocyte suspensions of T cell subsets, it was apparent that whereas UV/trioxsalen-inactivated VV stimulated only CD4⁺ T cells, VV-infected P815 cells stimulated only CD8⁺ T cells (data not shown). To ensure that the IFN-γ release was VV-specific, either extracts purified from uninfected cells and then treated with UV/trioxsalen as for inactivation of virus, or uninfected P815 were used as negative control antigens. All other reagents and conditions were as published (Schneider et al., 1998).

The ELISPOT technique was used to measure CD4⁺ and CD8⁺ T cell responses in groups of three mice (Fig. 3 a and b, respectively). Mice inoculated with 10⁴, 10⁵ and 10⁶ p.f.u. of VV had similar numbers of VV-specific CD4⁺ T cells in their spleens at all times tested and the same was true for CD8⁺ T cells. T cell responses were maximal during the peak of infection reflecting the large number of effector cells generated to clear the acute infection. Spleens were also enlarged visibly at these times and often had twice as many cells as spleens from uninfected controls. In this experiment, memory responses declined slightly from 24 to 49 days p.i. but in other experiments, VV-specific IFN-γ secreting T cells have been found to be maintained at 30/10⁶ splenocytes at 75 days p.i.
same levels of antibodies were found in mice inoculated with sulphuric acid and the optical density was read at 492 nm. The antibody titres (horseradish peroxidase. After development with the error bars show the range.

splenocytes in groups of three mice. Antibody levels were determined by b and c T cells in the sera of infected mice. Anti-VV antibody in the sera of infected mice was measured

day 5 day 24 day 49

time after infection

(a) CD4+ T cells

(b) CD8+ T cells

Fig. 3. Immune response to ear pinna infection. Numbers of VV-specific, IFN-γ-secreting CD4+ (a) and CD8+ (b) T cells in the spleens and anti-VV antibody titres (c) in the sera of mice inoculated with 10^4 (open bars in a and b; ▲ in c), 10^5 (hatched bars in a and b; ■ in c) and 10^6 (grey bars in a and b; ● in c) p.f.u. of VV. T cells were enumerated using ELISPOT assays on the days p.i. indicated below the horizontal axis. The bars represent means and range of numbers of spot forming cells (sfc)/10^6 splenocytes in groups of three mice. Antibody levels were determined by ELISA 20 days p.i.; each data point is the average titre from two mice and the error bars show the range.

was consistent with T cell responses, indicating that over the range of doses used, the immune response was independent of the size of the inoculum. This attribute of the model might be advantageous as it will tend to counter simple dose effects in experiments designed to compare the immunogenicity of VV mutants.

In conclusion, we report the characterization of a cutaneous model of VV infection that allows many facets of pathogenesis and immunity to be examined. Intradermal inoculation of ears with VV causes far less distress to the mice than intracranial and intranasal models but as with other cutaneous models, is sensitive to virus dose and genotype (Briody, 1959; Buller, 1985; Lee et al., 1992) (our unpublished results). In addition, the ear infection is highly localized to the site of inoculation while stimulating cellular and humoral immune responses, providing a good model for vaccination.

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References


Anti-VV antibody in the sera of infected mice was measured using standard ELISA techniques. VV-specific IgG was captured in wells coated overnight with UV/triolsalen-inactivated VV IMV (originally equivalent to 10^6 p.f.u. in 50 µl per well) and detected using rabbit anti-mouse IgG conjugated to horseradish peroxidase. After development with o-phenylenediamine dihydrochloride the reaction was stopped with sulphuric acid and the optical density was read at 492 nm. The same levels of antibodies were found in mice inoculated with 10^4, 10^5 or 10^6 p.f.u. per ear, 20 days p.i. (Fig. 3c). This result...


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