

Dermal infection with vaccinia virus reveals roles for virus proteins not seen using other inoculation routes

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Previously, we developed a model for testing the virulence and immunogenicity of vaccinia virus (VV) mutants based on the intradermal injection of BALB/c mouse ear pinnae. The model is characterized by a local infection in the inoculated skin without signs of systemic illness, mimicking dermal vaccination with VV. Here a further characterization of this model is presented, including the responses of mice to infectious virus doses as low as 10 p.f.u., a quantification of the infiltrate at the site of infection and use of different virus and mouse strains. The model was then used to compare the pathogenesis of six mutants of VV strain Western Reserve (WR) lacking genes A36R, A40R, A44L, B12R, B13R or B15R with that of appropriate control viruses. All of these genes except B12R and B15R influence the outcome of dermal infection with WR and for A40R and B13R this is the first role that has been reported after infection of mammals. A comparison of new and published results from intradermal and intranasal models is presented, showing that out of 16 gene deletion or insertion mutants of VV, half have phenotypes distinct from controls in only one of these models. Thus, the intranasal and intradermal models are complementary tools for dissecting the genetic basis of VV virulence.

Introduction

Vaccinia virus (VV) is the prototype poxvirus and the live vaccine used to eradicate smallpox (Fenner *et al.*, 1988). Within 2 years of the certification of smallpox eradication, VV recombinants expressing foreign genes had been constructed, raising the possibility that VV might be used as a vaccine against other infectious diseases (Panicali *et al.*, 1983; Smith *et al.*, 1983). Although several VV-based vaccines are in clinical trials, none have been licensed yet for human use. A major concern has been vaccine safety because it was recognized that vaccination with VV could cause complications, especially in immunocompromised individuals (Fenner *et al.*, 1988). Studies

of VV virulence have identified many VV proteins that modify the host response to infection (Smith *et al.*, 1997; Alcamí & Koszinowski, 2000; Moss & Shisler, 2001), and manipulation of these genes may enable the design of safer and more immunogenic VV strains. Several highly attenuated or replication defective strains of VV are being developed as candidate vaccines, such as modified virus Ankara (MVA) (Antoine *et al.*, 1998) or NYVAC (Tartaglia *et al.*, 1992), but these VV strains still encode some immunomodulators (Antoine *et al.*, 1998; Blanchard *et al.*, 1998). To determine which VV genes should be deleted to make better vaccines the role of each candidate gene should be characterized *in vitro* and *in vivo*.

The origin and natural host of VV remain obscure (Baxby, 1981) and consequently several different animal models have been used to study VV virulence. Mice have been infected with VV by the intranasal (i.n.), intraperitoneal (i.p.), intracranial (i.c.) and intradermal (i.d.) routes. The i.n. route has been used most widely, often being used exclusively to determine the virulence of VV mutants (Turner, 1967; Williamson *et al.*, 1990). However, there are several VV genes that do not alter virus virulence in this model, but which are conserved across a wide range of VV strains and therefore are likely to encode

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important viral functions. I.c. infection has been a sensitive route for distinguishing between VV mutants, but is less physiologically relevant than the i.n. route and the outcome of infection by the i.n. and i.c. routes shows a similar trend (Briody, 1959; Turner, 1967; Lee *et al.*, 1992). Furthermore, in at least some cases, neurovirulence in mice is a poor indicator of safety in humans (Williamson *et al.*, 1990).

We developed an i.d. model of VV infection in BALB/c mouse ear pinnae to provide a better model for vaccination (Tschärke & Smith, 1999). After i.d. inoculation of up to 10^6 p.f.u. of VV strain WR, virus replication remained local and a lesion was formed on the inoculated ear pinna, but no signs of generalized illness were seen. The size of the lesion served as a consistent measure of virulence and was sensitive to the dose of virus administered (Tschärke & Smith, 1999). In addition, the deletion of some VV genes affected disease in the i.d. model, but these deletions were not necessarily also associated with altered virulence after i.n. infection (Price *et al.*, 2000; Gardner *et al.*, 2001; Ng *et al.*, 2001). For this reason we explored the utility of the i.d. model further. We report here the results of our further characterization of the i.d. model as well as its application to a panel of seven VV strains, two other orthopoxviruses and six single gene deletion mutants of VV.

Methods

■ **Mice.** Female BALB/c mice, 6 to 18 weeks old, were bred at the Sir William Dunn School of Pathology (Oxford, UK). Female C57BL/6 and CBA mice, 6 weeks old, were purchased from Harlan (Bicester, UK). Mice were maintained in specific pathogen-free conditions.

■ **Viruses and cell lines.** All viruses were grown in either TK⁻143B or RK₁₃ cells and were titrated by plaque assay on BS-C-1 cells as described (Mackett *et al.*, 1985). The infectivity of each virus inoculum remaining after infection of animals was re-titrated to check the dose administered. The VV strains used in this study have been published previously (Alcami & Smith, 1995). VV strain Western Reserve (WR) mutants lacking genes A36R (Parkinson & Smith, 1994), A40R (Wilcock *et al.*, 1999), A44L (A44L::ecogpt = vJM2; Moore, 1992; Moore & Smith, 1992), B12R (Banham & Smith, 1993), B15R (Alcami & Smith, 1992) and B13R (Kettle *et al.*, 1995) have been described previously.

■ **Infections.** Groups of five to nine mice were anaesthetized by inhalation of fluothane and the left ear pinna was spread over the operator's thumb, shielded with an inside-out rubber-thumb. The tip of the ear was held against the thumb by the forefinger and the mouse lifted so that the ear lobe was stretched over the thumb. The exposed (dorsal) surface of the left ear pinna was then injected with 10 μ l of virus suspension using a 27-gauge needle and 100 μ l glass syringe (Hamilton Company, Reno, NV, USA). A bubble was raised as the fluid was injected. Mice were examined daily and the diameter of lesions at the inoculation site were estimated to the nearest 0.5 mm using a micrometer. Any visible lesion was scored at least 0.5 mm. If more than one lesion was seen, or if lesions were obviously elongated, an estimation of the diameter of a single round lesion large enough to contain all the affected areas was made. Lesions on the ventral side of lobes were scored as above, but if both sides were affected, only the larger diameter was recorded. This system of inoculation and estimation of lesion size has proved to give consistent results with more than one operator and mice from different breeding colonies.

■ **Extraction of virus and cells from ears.** At various times post infection (p.i.) mice were killed and the left ear pinna was removed with dissecting scissors and placed in 1 ml of minimal essential medium (MEM). For virus titrations, samples were frozen and stored at -70°C until needed. Thereafter, samples were thawed, ground using 1 ml tissue grinders (Wheaton, Millville, NJ, USA), sonicated, and subjected to two further cycles of freezing and thawing and a final sonication before the virus infectivity was titrated by plaque assay on BS-C-1 cells.

To count the number of infiltrating cells, ears and the medium they were collected in were placed in a 6 mm dish. The two leaflets of the pinna were teased apart using fine forceps and the inside surfaces were scraped lightly with a rounded scalpel blade to remove cells in the dermis. The cells were centrifuged and resuspended in a solution containing 0.15 M NH_4Cl , 1 mM KHCO_3 and 0.1 mM Na_2EDTA to lyse red blood cells. After a wash in MEM, the remaining cells were passed through a 70 μ m nylon cell strainer (BD Falcon, NJ, USA) and counted using a haemocytometer.

■ **Measurement of anti-VV IgG by ELISA.** Blood was collected by cardiac puncture of euthanized mice and was allowed to clot overnight at 4°C . The next day the sample was spun at $> 10000\text{ g}$ for 10 min and the serum was decanted. Wells of a 96-well plate were coated overnight with the equivalent of 2×10^6 p.f.u. of intracellular mature VV (rendered non-infectious by treatment with psoralen and longwave ultraviolet light; Hanson *et al.*, 1978) in 50 μ l of PBS. Sequential 4-fold dilutions of serum were prepared in these wells and were incubated for 30 min at room temperature. After washing three times with PBS, bound IgG was detected by incubation with a 1:5000 dilution of rabbit anti-mouse IgG conjugated to horseradish peroxidase (Serotec). Wells were washed three more times and the assay was developed with 100 μ l of *o*-phenylenediamine dihydrochloride (OPD complete tablet set, Sigma) for 15 min and stopped by addition of 25 μ l of 1 M sulphuric acid. The absorbance was read at 492 nm and the dilution of serum giving a half maximal density was calculated and recorded as the titre.

Results

Infection with low doses of VV

In BALB/c mice, although the lesion size was related to the inoculated dose of VV WR, the peak titre of virus infectivity in the ears was equivalent over a range of inocula from 10^4 to 10^6 p.f.u. (Tschärke & Smith, 1999). In an attempt to find a dose of virus that does not produce the maximum possible virus titre in the skin, lower doses of VV WR (10^2 or 10^3 p.f.u.) were inoculated and the infectious virus in the ears was measured at the indicated times p.i. (Fig. 1a). All the mice inoculated with 10^3 p.f.u. had visible lesions by 6 days p.i., whereas some mice given the lower dose only acquired lesions at 8 days p.i. The average diameters of lesions between days 5 and 8 are shown in Fig. 1(b). In contrast to the differences in lesion size, the maximum titres of virus in infected ears was similar irrespective of the dose administered. By 5 days p.i. all mice had titres of 10^6 p.f.u./ear, at 8 days p.i. titres were still 10^6 p.f.u., and at 11 days p.i. only one of four mice showed a substantial clearance of virus. In a further experiment, five mice were inoculated with 10 p.f.u. of WR and an average of 8.3×10^5 p.f.u. was found in the infected ears 10 days p.i., with one mouse harbouring 2.5×10^6 p.f.u. at this time. We conclude that there is a

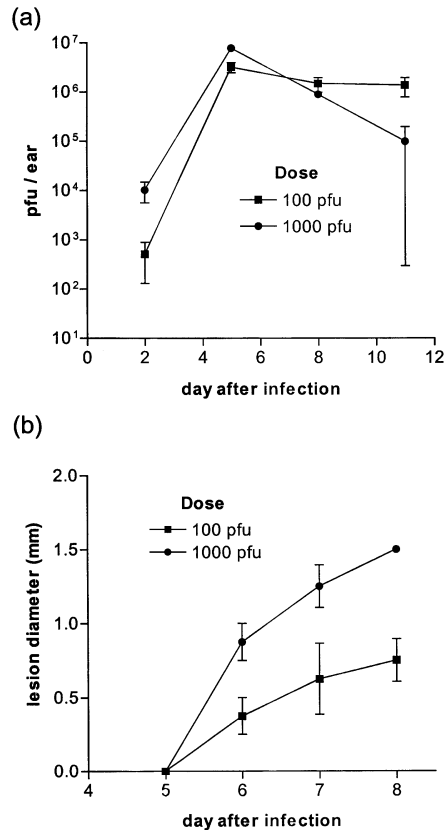


Fig. 1. Infectious virus titre (a) and lesion size (b) in the i.d. model. Groups of eight, female, BALB/c mice, 8 weeks old, were inoculated with 10^2 (■) or 10^3 (●) p.f.u. of VV strain WR and the titres of infectious virus in infected ear lobes (two mice per time-point) were determined at the times shown. Lesions were assessed on the mice killed on day 5 and the 4 remaining mice in each group between 6 and 8 days p.i. Points and error bars represent means and range of virus titres and mean and standard error of lesion sizes.

correlation between inoculation dose and lesion size for doses between 10^2 and 10^6 p.f.u., but there is no correlation between peak virus titres and inoculated dose or lesion size. The ability to use low virus doses increases the chances of observing minor differences in replicative capacity of VV mutants *in vivo*.

Extraction of infiltrating leukocytes

Sections of infected ear lobes contained a heavy cellular infiltrate that was maximal 12 days p.i., a time that corresponds to the peak size of lesions (Tschärke & Smith, 1999). To quantify this infiltrate, cells extracted from between the two layers of epidermis of infected ears were counted at various times p.i. (Fig. 2). At 3 days p.i., the number of cells extracted was only marginally higher than from the uninfected control, but by 6 days p.i. the number had risen to nearly 30-fold the uninfected amount, and at 9 days p.i. a further 4-fold increase was evident. The level and timing of infiltration correlated well with microscopic analysis (Tschärke & Smith, 1999), but the extraction method yielded significant debris making further analysis difficult.

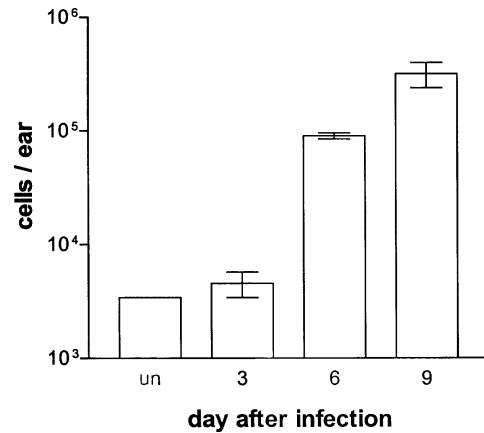


Fig. 2. Numbers of infiltrating cells recovered from ears of mice on the indicated days p.i. with 10^4 p.f.u. of VV strain WR. Bars represent the mean and range of two animals, except uninfected (un), which was a single mouse.

Effect of age of mice on lesion size

Observations from many experiments suggested that the age of mice might influence the size of lesions formed. Therefore, we infected groups of six, female, BALB/c mice that were 3, 6, 9, 12 and 18 weeks old, with members of each group being born within 8 days of each other, and lesions were measured daily (Fig. 3a). The age of mice had a very strong effect on lesion size, with 18-week-old mice having peak lesion sizes less than half that of 6-week-old mice. Mice of intermediate ages had lesion sizes between these two extremes, with younger groups always having larger lesions. The implications of this age effect are that groups of mice must always be age-matched, age ranges in groups should be minimized and the age of mice needs to be considered when comparing different experiments. For all subsequent experiments age ranges were kept to within 1 week.

Comparison of lesions in C57BL/6, CBA and BALB/c mice

Different inbred strains of mice often differ in their response to viral infections. We compared i.d. infection with VV WR in BALB/c mice with two other mouse strains, CBA and C57BL/6 (Fig. 3b). After infecting with 10^4 p.f.u., lesions were seen at the inoculation sites on all mice. The lesions on BALB/c and CBA mice were very similar, but C57BL mice had much larger lesions at most times. Thus the lesions associated with VV infection in the i.d. model are not restricted to BALB/c mice and C57BL/6 mice are much more susceptible to lesion formation than BALB/c or CBA.

Comparison of different strains of VV and other orthopoxviruses

Different strains of VV have varying levels of virulence in animal models and also in vaccinated humans (Fenner *et al.*, 1988), but the basis of this differential virulence is not

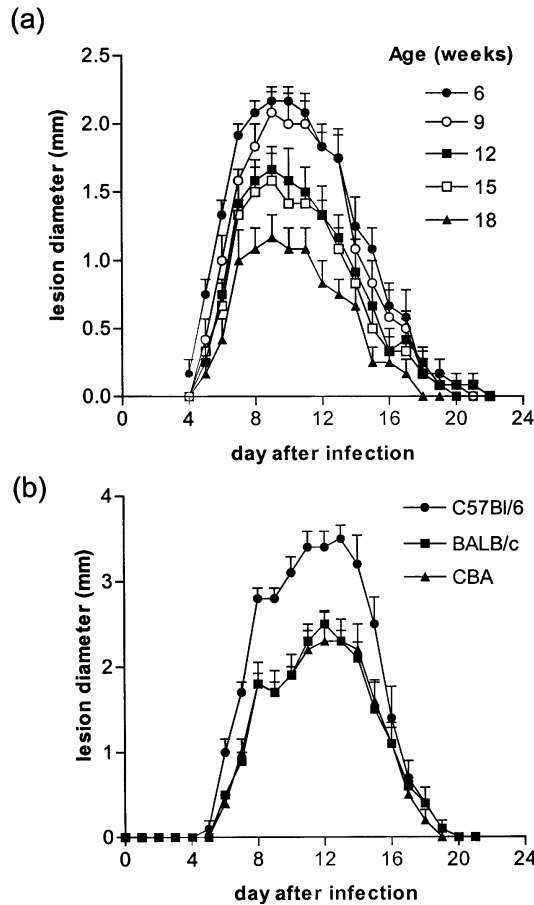


Fig. 3. Effect of age and strain of mice in the i.d. model. (a) Groups of five BALB/c mice of the ages indicated were infected with 10^4 p.f.u. of VV WR and lesion sizes were estimated daily. (b) Groups of five C57BL/6 (●), BALB/c (■) or CBA (▲) mice were infected with 10^4 p.f.u. of VV WR and lesion sizes were estimated daily. Points and error bars represent the mean and standard error for each group of five mice.

understood. Potential virulence genes can be studied in their original genetic backgrounds only if the VV strains that harbour them are able to cause disease in the animal model/s of choice. For this reason, and to investigate other VV strains in addition to the neurovirulent mouse-adapted WR strain (Parker *et al.*, 1941), we tested six other VV strains in the i.d. model. Groups of five, female, BALB/c mice (8 weeks old) were infected with 10^6 p.f.u. of VV strains IHD-J, WR, Tian Tan, Copenhagen, Tashkent, Lister and Wyeth and lesions were estimated daily (Fig. 4a). The strains fell into four groups with respect to their ability to cause lesions: (1) IHD-J caused by far the largest lesions, (2) WR, Tian Tan and Copenhagen formed a group having lesions of moderate size, (3) Tashkent and Lister produced small lesions over a shorter period and (4) Wyeth produced tiny lesions on only three of five mice in the group and these were only seen for a few days. With the exception of Wyeth that gave too mild a lesion in this model, all VV strains investigated should be amenable to analysis using the i.d. model after optimizing the dose.

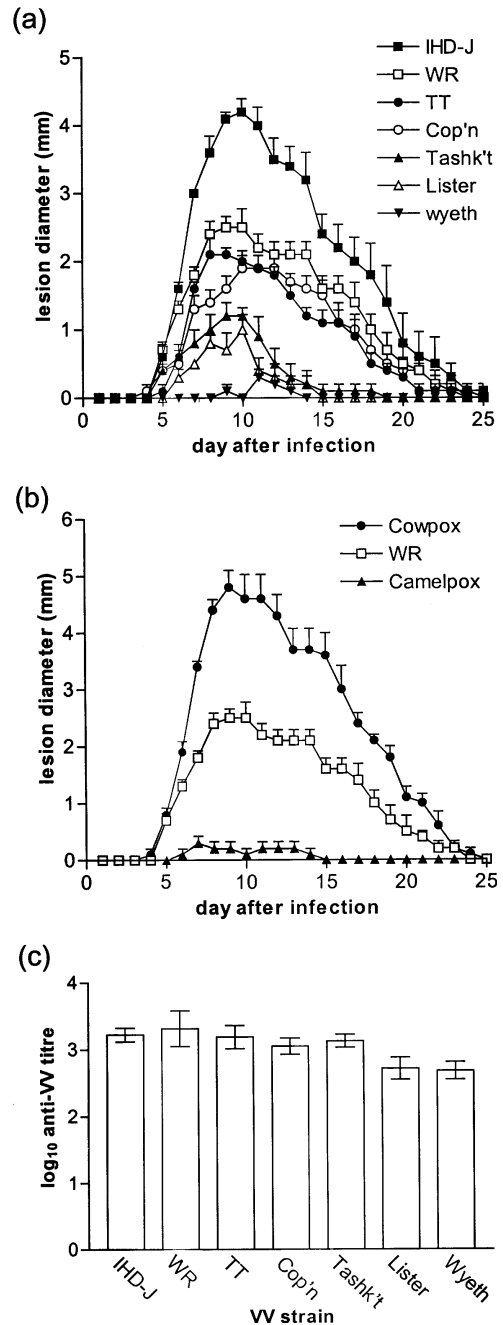


Fig. 4. I.d. infection with different strains of VV (TT, Tian Tan; Cop'n, Copenhagen; Tashk't, Tashkent) and other orthopoxviruses. (a) Groups of five BALB/c mice were infected with 10^6 p.f.u. of the VV strains shown and lesion sizes were recorded daily. (b) Groups of five BALB/c mice were infected with 10^6 p.f.u. of CPV (●) or CMPV (▲); data for VV strain WR from (a) (□) are included for comparison. For (a) and (b), points and error bars represent means and standard errors, respectively. (c) Anti-VV IgG response after i.d. infection with the VV strains shown. Sera from three of the mice from the groups in (a) were tested for anti-VV IgG by ELISA (as described in Methods). Open bars and error bars represent average titres and 95% confidence limits, respectively.

At the same time we tested two other orthopoxviruses, cowpox virus (CPV) strain Brighton Red and camelpox virus (CMPV) strain CM-S (Gubser & Smith, 2002), and the lesions

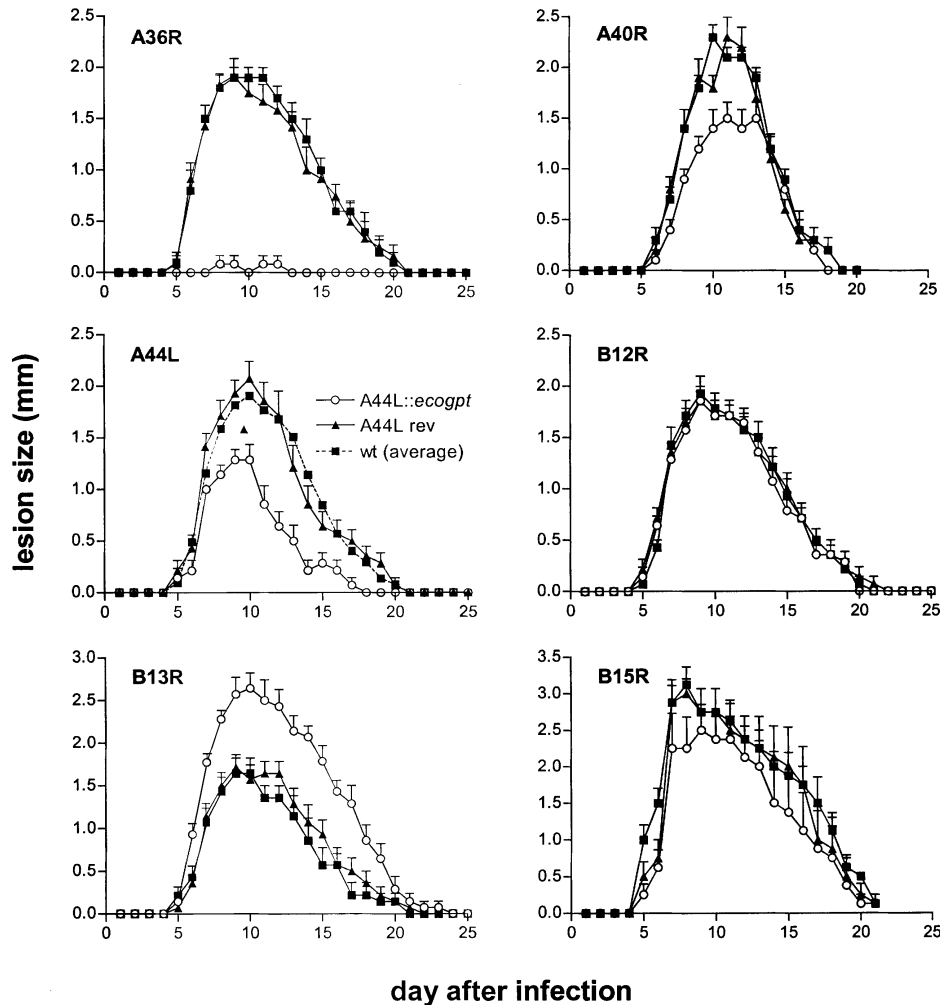


Fig. 5. Effect of single gene deletions from VV strain WR on lesion size. The gene deleted is indicated at the top-left corner of each graph. Groups of between five and seven mice were inoculated with 10^4 (or 10^6 for $\Delta B15R$ and control viruses) p.f.u. of a wild-type, deletion or revertant virus. For A44L, the viruses used were $\Delta A44L::ecogpt$ and revertant as shown; the wt line on this graph (■, dotted line) is an average of data obtained with all four wt viruses shown in the figure and used at the same dose. Lesion sizes were estimated daily and means and standard errors are shown.

induced by these viruses are compared to WR in Fig. 4(b). CPV caused large haemorrhagic lesions that at their peak were twice the size of lesions produced by WR. In contrast, only three of five mice inoculated with CMPV had lesions and no lesions were larger than 0.5 mm.

The anti-VV serum Ab response following infection with these viruses was investigated 3 months after infection as described in Methods (Fig. 4c). There was very little variation in Ab titres amongst the groups infected with the different strains, although Lister and Wyeth, the two VV strains that produced the smallest lesions had 2- to 3-fold (significant at 95% confidence) lower titres than the other strains.

Analysis of a panel of single gene deletion mutants of VV

The i.d. model has demonstrated a phenotype for mutant VVs lacking genes B7R, A39R and A41L (Price *et al.*,

2000; Gardner *et al.*, 2001; Ng *et al.*, 2001). To understand better the applicability of the model to other mutants and to investigate the roles of other VV genes we tested a range of deletion mutants (Δ) of VV strain WR along with their relevant control wild-type (wt) and revertant (rev) viruses in the i.d. model. Mutants selected included those lacking genes A36R, A40R, A44L, B12R, B13R and B15R, all of which have been examined in the i.n. model allowing comparisons to be made between the i.n. and i.d. models. Age matched, female, BALB/c mice were infected with 10^4 p.f.u. (or 10^6 p.f.u. for the B15R virus set) of wt, Δ or rev viruses [or insertional inactivation ($\Delta A44L::ecogpt$) and rev for A44L] and the lesion sizes at the inoculation site were estimated daily (Fig. 5). Three mutants produced lesions that were significantly smaller, $\Delta A36R$ ($P < 0.001$) and $\Delta A40R$ ($P < 0.01$) or larger, $\Delta B13R$ ($P < 0.001$) than relevant wt and rev viruses as assessed by *t*-test on at least one day. In addition, $\Delta B15R$ produced lesions

that were consistently smaller than wt and rev viruses over most of the course of infection, but were not significantly different on any single day. This result has been reproduced for the first 12 days p.i. with a dose of 10^4 p.f.u. (data not shown), but because of the lack of statistical significance in both experiments we cannot conclude that B15R expression affects lesion size. A44L::ecogpt is a mutant lacking a functional A44L gene and produced smaller lesions ($P < 0.01$) than its revertant, but a matched wt was not available for direct comparison with these viruses. However, combined data from all four other wt viruses used here at the same dose (10^4 p.f.u.) produced a time-course of lesion sizes (wt average) that was significantly different when compared with those from mice infected with A44L::ecogpt ($P < 0.01$, on 7 days), but not A44L rev ($P > 0.1$, all days). Therefore, we conclude that A44L expression from VV WR affects lesion size. The remaining mutant, Δ B12R, did not cause lesions that were different from those caused by its wt and rev controls.

We also examined the humoral response after infection with some of the deletion viruses that differed in lesion forming ability compared with controls. Sera taken from mice 3 months after infection with the A36R, B13R and B15R sets of viruses were tested for anti-VV Ab titre. Although Δ A36R was unable to cause lesions, it induced an Ab titre only 2- to 2.5-fold lower (but significantly different at 95% confidence) than its wt and rev controls. No significant differences in titres were seen amongst the other sets of viruses (data not shown).

Discussion

This report provides a further characterization of the mouse i.d. model for studying the virulence of VV and possibly other orthopoxviruses. It also establishes roles in virulence for two VV genes, A40R and B13R, not seen in the i.n. model. We investigated a wider range of inoculation doses, different ages and strains of mice, different VV strains, CMPV and CPV, and six VV strain WR mutants from which individual genes have been deleted. Data presented show that very low doses of virus still produce high titres of infectious virus (approximately 10^6 p.f.u.), but that the lesion size is related to the dose administered. The model showed variation in the virulence of VV strains that mirrors the virulence of these viruses as smallpox vaccines in man (Fenner *et al.*, 1988), where these had been used.

Several models have been used for studying poxvirus pathogenesis, such as ectromelia virus and CPV in mice, myxoma virus in the European rabbit and VV in mice (Buller & Palumbo, 1991). In the case of ectromelia virus (Fenner, 1948) and cowpox virus (Chantrey *et al.*, 1999) the use of a mouse model is appropriate since these viruses are natural pathogens of rodents. With myxoma virus the natural host is the South American rabbit in which infection is often asymptomatic, whereas the host used for studies of myxoma virus virulence is

the European rabbit in which the virus causes the disease myxomatosis (Fenner & Ratcliffe, 1965). In contrast to these viruses, the origin of VV is unknown and so the most appropriate animal model for studying VV virulence is uncertain. However, mouse models have been used to uncover potential roles for a large number of VV genes (Smith *et al.*, 1997). Furthermore, it cannot be assumed that orthologous genes act in a similar way in different virus genetic backgrounds (see below); thus work with other orthopoxviruses cannot be relied upon to find virulence genes in VV. Much of the work on VV virulence in mice has been done using inoculation routes such as i.c., i.p., and i.n. that cause systemic infection. In contrast, the i.d. model used here is characterized by a milder local infection, without signs of general illness. These features mimic dermal vaccination and make the i.d. model, but not the i.c., i.n. or i.p. models, suitable for studies relating to vaccine development.

Another attractive feature of the mouse i.d. model is the wide range of doses of infectious virus that can be used. Here we show that after inoculation of only 10 p.f.u. of WR virus, titres of over 10^6 p.f.u. can be found in the ear a few days later. The ability to use very low doses of virus maximizes the chance of observing phenotypic differences between strains of virus that might have only slight differences in their capacity to replicate. It is not clear why infection with very different virus doses leads to similar peak virus titres; perhaps in the thin epidermis of the ear lobe the number of available host cells is limiting. Despite the similarities in peak virus titres, the size of the lesions induced remained proportional to the inoculum dose used. These observations suggest that the nature of the early inflammatory response to infection may be important in determining lesion size in VV infection in this model. Innate defences such as interferons, complement and cytokines may influence immunopathology, as could the various types of inflammatory leukocytes recruited to the lesion. We have quantified the total number of cells infiltrating into VV-induced dermal lesions using a scraping method. To reduce the amount of cell debris, a new method based on Belkaid *et al.* (1996) is being developed to enable quantification of the cell types present at the site of infection. This might reveal differences in the infiltrates induced by wt and mutant viruses.

The age of mice affects the lesion size with younger animals developing larger lesions (Fig. 3a), yet all mice used would be expected to be immunocompetent. One difference between the ears of young and old mice is the relative softness of the skin in younger mice, so possibly the tougher skin of older mice is less susceptible to viral and/or immunopathological damage. We have not tested whether the larger lesions are associated with higher titres of virus.

The strain of mouse used also influenced lesion size. Lesions formed in BALB/c and CBA mice were equivalent, whereas larger lesions were formed in C57BL/6 (Fig. 3b). This result is in contrast to the susceptibility of these strains to infection with ectromelia virus, to which C57BL mice are resistant due to

stronger and/or earlier responses by both innate and adaptive immune mediators (Buller & Palumbo, 1991). However, these findings are in agreement with an early report by Briody (1959), in which C57BL/6 mice were found to allow greater replication of virus in the skin than most mouse strains after dermal scarification, and yet were more resistant to i.n. infection when compared with these same strains. Whether the larger lesion size on C57BL mice shown here is a result of increased virus growth or immunopathology due to stronger immune responses needs to be shown.

Different VV strains cause widely different lesions (Fig. 4a), but mostly the lesions are large enough to allow analysis of mutations in each virus genetic background. IHD-J produces particularly large lesions and *in vitro* forms a normal size plaque but enhanced levels of extracellular enveloped virus (Payne, 1980) due to a mutation in the A34R gene (Blasco *et al.*, 1993; McIntosh & Smith, 1996). Interestingly, Wyeth, WR and IHD-J caused very different lesion sizes and yet were all derived from the New York City Board of Health (NYCBH) strain. WR and the IHD strains were passaged in mouse brain and are more virulent in mice than NYCBH after i.c. inoculation (Parker *et al.*, 1941), but the production of larger lesions by IHD-J compared to WR virulence was unexpected because in the i.n. model these viruses both spread to the brain and induced similar mortality (Payne, 1980). CMPV gave very small lesions, although limited replication may still have occurred. In contrast, CPV strain BR produced very large lesions that were haemorrhagic, unlike all VV lesions except some caused by IHD-J (data not shown). Ear pinna injections with an appropriate dose of CPV would cause less distress to mice and might prove to be a useful addition to the more commonly used footpad model of CPV (Miller *et al.*, 1995). We have not tested whether CPV spread is as highly restricted in this model as VV WR, although we noted no signs of systemic illness.

Despite the wide range of lesion sizes, similar anti-VV Ab titres were produced. In fact, even VV strain MVA produced Ab titres similar to WR if doses of $\geq 10^6$ p.f.u. were used (data not shown). At these doses the level of antigen produced seems sufficient for a good humoral response, but following i.n. infection, which gives a systemic infection, Ab titres are significantly higher (data not shown and Briody, 1959). In a study by Lee *et al.*, (1992), which used dermal scarification, Ab responses to Wyeth were compromised at low doses and in general Ab responses were proportional to virus titres. The slightly lower anti-VV Ab titres following infection with Lister, Wyeth, and $\Delta A36R$ may be due to reduced virus replication or spread.

The range of VV genes known to affect virulence in the i.d. model has been extended by the study of six deletion mutants and their controls. The phenotypes of these mutants included increased virulence ($\Delta B13R$), attenuation ($\Delta A36R$, $\Delta A40R$ and $\Delta A44L$) and no significant difference ($\Delta B12R$, $\Delta B15R$). B13R (also called SPI-2) is a member of the serpin family of protease

inhibitors and $\Delta B13R$ was the only mutant with increased virulence. The related CPV protein (crmA) has 92% amino acid identity, is an intracellular inhibitor of caspase 1 (Ray *et al.*, 1992) and affects the pock phenotype of CPV on the chorioallantoic membrane (Pickup *et al.*, 1986; Palumbo *et al.*, 1989). VV B13R protein also inhibits caspase 1 preventing the apoptosis of infected cells in response to TNF or fas ligand/fas interaction, and the processing of pro-IL-1 β (and presumably pro-IL-18) into their mature forms (Dobbelstein & Shenk, 1996; Kettle *et al.*, 1997). Despite these positive findings *in vitro* for B13R of VV and *in vivo* for crmA in CPV, a role in VV virulence was not found for B13R using deletion and revertant viruses in the i.n. model (Kettle *et al.*, 1995). A report that deletion of SPI-2 (B13R) from rabbitpox virus caused attenuation in the murine i.n. model did not include a revertant virus (Thompson *et al.*, 1993) and it was discovered subsequently that the deletion mutant contained an additional mutation elsewhere in the genome (R. A. Moyer, personal communication). In contrast, the i.d. model revealed a role for B13R in VV pathogenesis, but surprisingly, removing the gene enhanced pathology. In fact, the large lesion phenotype of $\Delta B13R$ in the i.d. model was more striking than for any other WR mutant we have studied and remains to be reconciled with the *in vitro* studies.

The greatest attenuation was observed with $\Delta A36R$, a result consistent with the striking attenuation seen with this virus in the i.n. model (Parkinson & Smith, 1994) and the failure of this virus to make actin tails and to spread efficiently from cell to cell in culture (Sanderson *et al.*, 1998; Wolffe *et al.*, 1998; Röttger *et al.*, 1999; van Eijl *et al.*, 2000). A more modest attenuation was seen with $\Delta A40R$, a virus that had showed no attenuation in the i.n. model (Wilcock *et al.*, 1999). This represents the first report of a phenotype for A40R *in vivo*, and could perhaps reflect an immunomodulatory role for this protein given its similarity to C-type lectins including natural killer cell receptors, the human IgE receptor and CD69, an early activation marker on lymphocytes. Another gene that promoted lesion size in the i.d. model was A44L. This gene encodes 3 β -hydroxysteroid dehydrogenase, a biosynthetic steroid enzyme associated with virulence in the i.n. (Moore & Smith, 1992) and i.c. models (Sroller *et al.*, 1998). The early production of immunosuppressive steroids by A44L may dampen the early immune response to the virus, thereby promoting replication and spread.

A comparison of results from experiments with VV strains and single gene deletion mutants of WR, combined with knowledge of the distribution of these genes across VV strains, highlights the complexity of the genetics of poxvirus pathogenesis. For example, deletion of B13R from WR results in a virus that produces very large lesions after i.d. inoculation, but VV strains Copenhagen, Tian Tan, Tashkent and Lister all lack B13R and yet produce lesions of a size similar to, or smaller than, WR. IHD-J on the other hand expresses B13R but not A40R, a combination that would be expected to reduce lesion

Table 1. VV strain WR genes investigated in i.d. and i.n. models

Gene	Function	Effect of deletion		Reference*
		Intradermal	Intranasal	
A36R	Essential for actin tails	Tiny lesion	Less virulent	Fig. 4; Parkinson & Smith (1994)
A41L	Modulator of immunopathology	Larger lesion	Nil	Ng <i>et al.</i> (2001)
A44L	3- β -Hydroxysteroid dehydrogenase	Smaller lesion	Less virulent	Fig. 4; Moore & Smith (1992)
A40R	Similar to animal C-type lectins	Smaller lesion	Nil	Fig. 4; Wilcock <i>et al.</i> (1999)
A45R	Unknown	Nil	Nil	Almazán <i>et al.</i> (2001)
B7R	Unknown	Smaller lesion	Nil	Price <i>et al.</i> (2000)
B8R	IFN- γ binding protein (not mouse)	Nil	Nil	Alcamí & Smith (1995, 2002); Symons <i>et al.</i> (2002)
B9R	Unknown	Nil	Nil	Price <i>et al.</i> (2002)
B12R	Unknown	Nil	Nil	Fig. 4; Banham & Smith (1993)
B13R	Serpin, SPI-2	Larger lesion	Nil	Fig. 4; Kettle <i>et al.</i> (1995)
B15R	IL-1 β binding protein	ns	More virulent	Fig. 4; Alcamí & Smith (1992, 1996)
C2L	Unknown	Larger lesion	Nil	B
C12L	IL-18 binding protein	Nil	Less virulent	A
N1L	Unknown	Smaller lesion	Less virulent	Kotwal <i>et al.</i> (1989); Bartlett <i>et al.</i> (2002)
Effect of insertion				
vCKBP	CC chemokine binding protein	Nil	Less virulent	A; Alcamí <i>et al.</i> (1998)
A39R	Semaphorin	Larger lesion	Nil	Gardner <i>et al.</i> (2001)

* References: A, unpublished data, Julian A. Symons, DCT; B, unpublished data, Marta Pires de Miranda, DCT, PCR. ns, No significant difference.

sizes in WR, but this strain causes very large lesions (Figs 3 and 4, Kettle *et al.*, 1995; Wilcock *et al.*, 1999). A further example is the semaphorin A39R, which was found to affect lesion sizes when inserted into WR, but not when deleted from Copenhagen (Gardner *et al.*, 2001). Therefore, an *in vivo* function for a gene in one VV strain cannot be assumed for other strains, or orthopoxviruses in general.

Finally, we emphasize the importance of using more than one model when studying VV virulence. Table 1 lists all the VV genes examined using both i.d. and i.n. models and of 16 genes listed, half are associated with distinct phenotypes in only one of the models. Although roles in virulence have not been found for some conserved genes, the number of such genes has been reduced significantly through the use of the i.d. model. It is well established that the outcome of virus infection depends upon the route of inoculation; however, this study demonstrates clearly, and with many mutants, the independent utility of two different routes of inoculation in assigning roles in virulence to virus genes. We conclude that the i.d. model is a useful addition to the tools available for studying the virulence and immunogenicity of VV and perhaps other orthopoxviruses.

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