THE IMMUNE RESPONSE TO VACCINIA VIRUS IS SIGNIFICANTLY REDUCED AFTER SCARIFICATION WITH TK⁻ RECOMBINANTS AS COMPARED TO WILD-TYPE VIRUS

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Summary. – Although it is unlikely that large-scale vaccination against smallpox will ever be required again, it is conceivable that the need may arise to vaccinate against a human orthopoxvirus infection. A possible example could be the emergence of monkey poxvirus (MPV) as a significant human disease in Africa. Vaccinia virus (VV) recombinants, genetically modified to carry the immunogenic proteins of other pathogenic organisms, have potential use as vaccines against other diseases present in this region. The immune response to parental wild-type (wt) or recombinant VV was examined by binding and functional assays, relevant to protection: total IgG, IgG subclass profile, B5R gene product (gp42)-specific IgG, neutralizing antibodies and class 1-mediated cytotoxic lymphocyte activity. There was a substantial reduction in the immune response to VV after scarification with about 10^8 PFU of recombinant as compared to wt virus. These data suggest that to achieve the levels of immunity associated with protection against human orthopoxvirus infection, and to control a possible future outbreak of orthopoxvirus disease, the use of wt VV would be necessary.

Key words: vaccinia virus; immunity; TK ; scarification

Introduction

Smallpox has been eradicated worldwide (Fenner *et al.*, 1988) and it seems unlikely that mass vaccination will ever again be required to control naturally occurring orthopoxvirus infections of humans. The attenuated vaccine virus used in the smallpox eradication campaign, VV is now widely used as a vaccine vector, carrying immunogenic proteins from a variety of pathogens (Moss, 1996). In order that these putative vaccines may find acceptance by modern vaccine licensing authorities, it will be important for the manufacturers to use strains of VV known to be safe. These strains must not be associated with a significant incidence of post-vaccinal encephalitis, a severe and life-threatening complication of vaccination that principally affects children under 1 year old (Fenner *et al.*, 1988).

MPV infection is a rare disease, and natural outbreaks have so far only been reported in western and central Africa. The illness is clinically virtually indistinguishable from smallpox (Jezek and Fenner, 1988). There is little evidence of human to human spread, and the virus does not persist in the human community. However, there are areas of concern over MPV infection (Cohen, 1997). It is possible that the eradication of smallpox, followed by the stopping of mass-vaccination in rural Africa, may create an ecological niche for MPV to fill. This situation may be exacerbated by the presence of significant numbers of immuno-compromised

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Abbreviations: AIDS = acquired immunodeficiency syndrome; APC = antigen presenting cells; BTV = bluetongue virus; CI = confidence interval; CTL = cytotoxic lymphocyte; EEVV = extracellular enveloped VV; EIA = enzyme immunoassay; E:T = effector:target cell; IMVV = intracellular mature VV; MEM = Eagle's Minimum Essential Medium; MOI = multiplicity of infection; MPV = monkey poxvirus; p.s. = post scarification; VEEV = Venezuelan equine encephalitis virus; VV = vaccinia virus; wt = wild-type

individuals (sufferers from acquired immunnodeficiency syndrome (AIDS), malnutrition or other chronic disease) who may be more susceptible to MPV infection. This effect could enhance the level of MPV in the environment, leading to increased human-to-human transmission and even mutation of the virus to a more infectious phenotype.

There is no evidence that these events are occurring (Breman and Henderson, 1998) but it would be beneficial if a side effect of immunization with VV recombinant vaccines, directed at diseases prevalent in Africa, were to confer immunity against other orthopoxvirus infections. VV recombinants are often prepared via insertion of a foreign gene into and inactivation of the VV thymidine kinase (TK) gene. VV TK⁻ mutants are reduced in virulence compared to wt virus (Buller *et al.*, 1985).

We examined the immune response to wt and TK⁻ recombinant viruses, prepared in a VV strain potentially acceptable to modern medicine, in detail. From these data we infer the level of protection, that may be expected against other orthopoxvirus infections, after scarification vaccination with TK⁻ as compared to wt VV.

Materials and Methods

Viruses and cell cultures. VV Lister (LIS) strain was obtained from the Swiss Serum and Vaccine Institute, Berne, Switzerland. RK-13 cells were cultured and maintained in Eagle's Minimum Essential Medium (MEM) by standard methods. VV IHD-J strain was obtained from Dr D. Ulaeto, CBD, Porton Down, UK.

Construction of VV recombinants. Plasmids containing the coding regions for the VEEV structural genes E3-E2-6K-E1, E3-E2-6K or 6K-E1 were a kind gift from Dr S.C. Jacobs, CBD, Porton Down, UK. The cloned Venezuelan equine encephalitis virus (VEEV) genes were inserted into the shuttle vector p1113. This vector allows the generation of recombinant VV by homologous recombination and insertion of the VEEV genes into the VV TK gene with dominant selection of recombinants by mycophenolic acid resistance (Falkner and Moss, 1988). Protein expression was under the control of the VV 7.5K promoter. TK⁻ recombinant viruses, selected after Lipofectin™ (Life Technologies, USA) transfection of VV-infected RK-13 cells, underwent 3 rounds of plaque purification before stock virus was prepared as described earlier (Mackett *et al.*, 1985; Bennett *et al.*, 1998).

Immunization of animals. Balb/c mice (Charles Rivers Laboratories) aged 8–12 weeks were vaccinated once with 10^8 PFU of wt or recombinant VV by scarification on the shaved dorsal surface. All the vaccinated animals produced a visible skin lesion. Blood samples were taken at days 20 and 40 post vaccination.

Enzyme immunoassays (EIAs) were performed by standard methods using a peroxidase-linked second antibody and tetramethyl benzidine as the chromogenic substrate (VV antigens), or an alkaline phosphatase-linked second antibody and an amplified substrate system (Gibco-BRL) used according to the manufacturer's instructions (B5R glycoprotein antigen, gp42). VV antigen was sucrose density gradient-purified and dissolved in phosphate-buffered saline pH 7.5 before use in coating microtiter plates. The B5R gene product (GST fusion protein, cloned and expressed in *Escherichia coli*, a kind gift from Dr D. Pulford, CBD Porton Down, U.K.), was dissolved in 50 mmol/l bicarbonate buffer pH 8.5 and used in coating plates. The IgG antibody subclass profile was determined as described by Gupta and Siber (1985).

Neutralization assays were performed to detect neutralizing antibodies to both extracellular enveloped VV (EEVV) and intracellular mature VV (IMVV; McClain *et al.*, 1997; Vanderplasschen *et al.*, 1997). IHD-J strain was used as the virus antigen in both assays. In the EEVV assay adapted from that described by Vanderplasschen *et al.* (1997) IMVV present in the virus antigen was neutralized by incubation for 1 hr at 37°C with an antiserum to 1/20 dilution of a rabbit heat-inactivated VV antiserum (kindly supplied by Dr D. Ulaeto, CBD, Porton Down, U.K.) that neutralized more than 95% of the IMVV activity. EEVV or IMVV antigens were mixed with an equal volume of heat-inactivated mouse serum dilution and incubated for 1 hr at 37°C before plaque assay for residual infectious virus on RK13 cells. Twenty to 50 plaques per plate were non-immune mouse serum controls. The dilution of serum that produced a 50% reduction in plaque numbers was calculated. A rabbit antiserum to VV IHD-J strain was used as a positive control to demonstrate neutralization of EEVV.

Cytotoxic lymphocyte (CTL) assay. Balb/c female mice (5 per group) were scarified with $10^{7.7}$ PFU wt or recombinant VV and sacrificed at day 7 post inoculation. Their spleens were removed and prepared using a standard method (Offit *et al.*, 1991) prior to co-culturing for 7 days with autologous VV-infected, irradiated spleen cells from non-immunized mice. Effector cells (from 3 individual mice) and antigen presenting cells (APC) (infected with VV, multiplicity of infection (MOI) of 10 PFU/cell were co-cultivated at a ratio of 1:2 and resuspended in RPMI-10 medium. Target cells (P815) were grown to confluency at 37°C and infected with VV virus at MOI of 10 PFU/cell or mock-infected. All cells were washed twice in RPMI-10 and resuspended at a final concentration of 5×10^4 cells/ml. Indirect immunofluorescence analysis confirmed that 90–98% of cells were infected after 6 hrs of incubation.

Putative effector cells from each culture were tested in a non-radioactive cytotoxicity assay (Cytotox 96™) as described earlier (Jones *et al.*, 1996). Target cells (100 µl) were added in triplicate to the wells of a 96-well plate at effector:target cell ratios (E:T) of 50:1, 25:1, and 12.5:1. Spontaneous target cell release was estimated in medium alone and maximum release by deliberate lysis of the target cells. The plates were centrifuged at 275 x *g.* for 4 mins prior to incubation at 37°C in 5% CO_2 for 18 hrs. After a further centrifugation aliquots (50 µl) of the media from all wells were transferred to fresh plates prior to addition of substrate (50 µl) to each well. The reaction was stopped after 30 mins of incubation at room temperature and the A_{492} was measured within 1 hr.

Specific cytotoxicity (%) for each E:T was calculated from the A values using the formula:

$$\text{Specific cytotoxicity} = \frac{(A_{exp} - A_{eff.\ spont.}) - A_{targ.\ spont.}}{A_{targ.\ max.} - A_{targ.\ spont.}} \times 100$$

where $A_{exp.} = A_{experimental}$, $A_{eff.\ spont.} = A_{effector\ spontaneous}$, and $A_{targ.\ max.} = A_{target\ maximum}$. Bluetongue virus (BTV) effector cells and

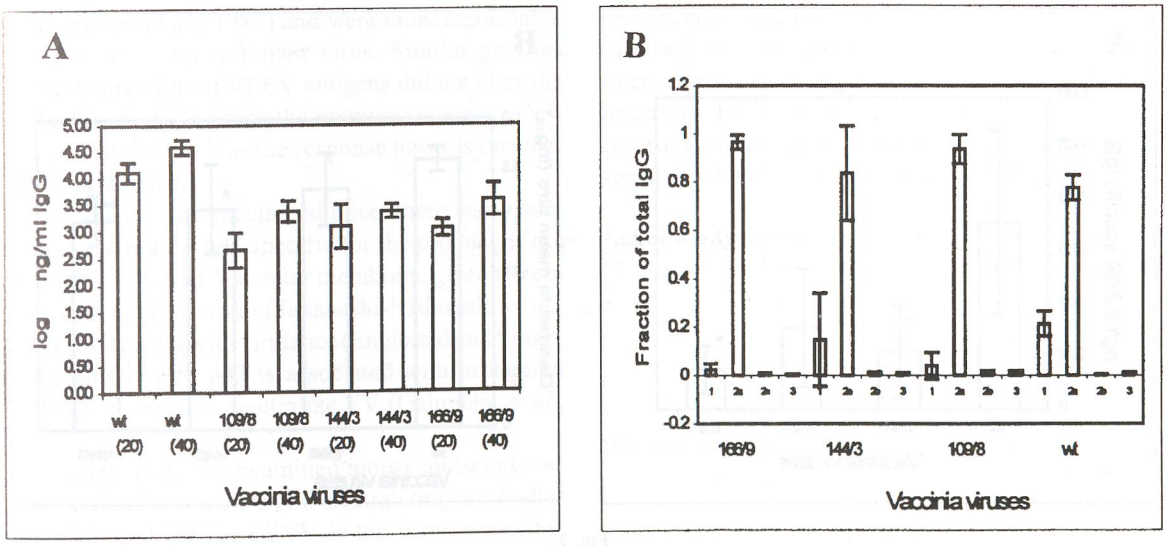

Fig. 1
VV-specific IgG

A: VV-specific IgG present in mouse sera (N = 10) on days 20 or 40 (day shown in parenthesis) p.s. with 10^8PFU of wt or recombinant VVs. B: IgG subclass distribution in mouse sera (N = 4) on day 40 p.s. Bar = 95% CI.

infected targets (positive controls) showed a variance of less than 8% (Jones *et al.*, 1996).

Statistical methods. An unpaired, 2-tailed t-test was used to estimate statistical significance of observed differences.

Results

IgG response

Previous experiments have indicated that the response to scarification of Balb/c mice with VV is maximal at day 40 post scarification (p.s.) (T. Lescott, unpublished data). The mean quantity of IgG in mouse serum at day 20 was 12.8 μg/ml rising to 38.9 μg/ml by day 40 (an approximate 3-fold increase). The mean level of serum IgG in all the recombinant groups was significantly ($p < 0.01$) lower than that in wt-generated sera, irrespective of day of sampling (Fig. 1A), but the levels for each recombinant were indistinguishable. The corresponding mean values for wt VV were 0.8 μg/ml (day 20) and 2.7 μg/ml (day 40). There was a similar, approximately 3-fold increase in IgG between days 20 and 40, but at both time points the total IgG level was 1/15 of that elicited by scarification with wt virus.

IgG 2a dominated the IgG subclass distribution of the response in both wt and recombinant-generated sera (Fig. 1B). This profile is typical of the response to live virus (Cohen, 1997). The profiles were the same on days 20 and 40 (data not shown).

Because of the important role played in the dissemination of infection by EEVV (Blasco and Moss, 1991, 1992; Payne, 1980) we measured IgG specific for the B5R gene product (gp42) a major EEVV outer membrane glycoprotein associated with virus neutralization and protective immunity (Galmiche *et al.*, 1988; Vanderplasschen *et al.*, 1997). The IgG response to gp42 was reduced in all the recombinant VVs in comparison to wt VV (Fig. 2A). The reduction was significant for recombinants 109/8 and 166/9 ($p < 0.05$, both comparisons) and approached the significance for recombinant 144/3 ($p = 0.11$).

Neutralizing antibodies

There was no neutralizing activity to EEVV in mouse sera on day 40 p.s. from the mice scarified with either recombinant or not VV (data not shown). There was a reduced neutralizing activity to IMVV in the recombinant VV-vaccinated mice as compared to the wt VV-vaccinated mice (Fig. 2B). The reductions were significant for recombinants 144/3 and 166/9 ($p < 0.05$ and $p < 0.01$, respectively) and approached the significance ($p < 0.1$) for recombinant 109/8.

CTL activity

There was a significant reduction in the CTL activity of spleenocytes of mice on day 7 p.s. with recombinant VV as compared to wt VV (Fig. 3) at all E:T ratios (recombinant 109/8), E:T ratios of 12.5:1 and 25:1 (recombinant 144/3), and 12.5:1 (recombinant 166/9).

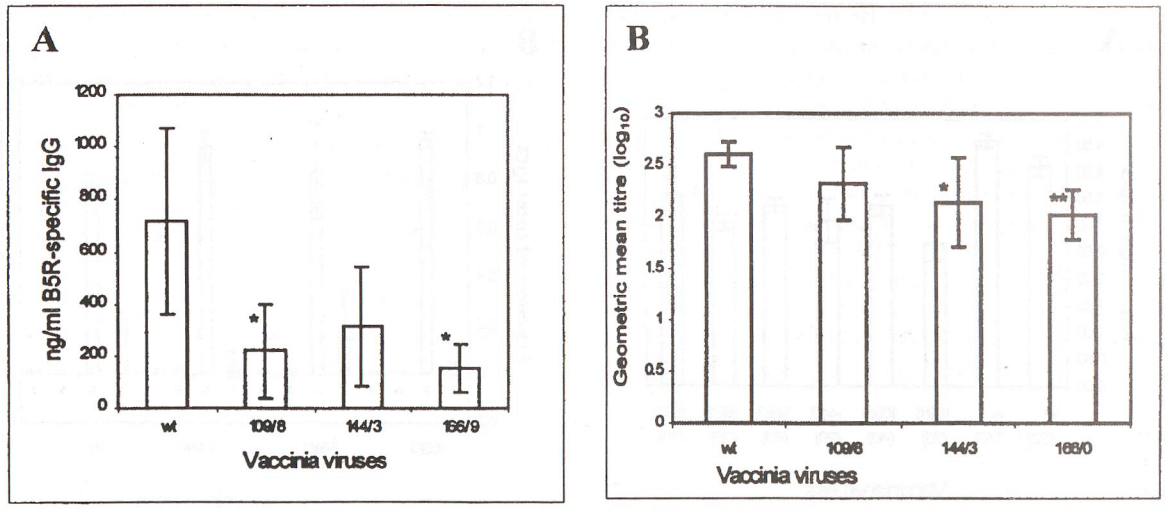

Fig. 2
B5R-specific IgG
A: Levels of B5R-specific IgG in mouse sera (N = 5) on day 40 p.s. with 10^8 PFU of wt or recombinant VVs. B: Neutralizing activity against IMVV in mouse sera. Bar = 95% CI. p <0.05 (*), p <0.01 (**) in comparison to wt VV.

Discussion

As smallpox has been eradicated worldwide a mass vaccination with VV is not only unnecessary but presents an unacceptable risk due to the albeit low frequency of vaccine complications. The waning of immunity to orthpoxvirus infections may present an opportunity for orthopoxviruses other than variola (specifically for MPV) to infect humans. In a population containing immuno-defficient individuals, severe infections may occur with the shedding of relatively large quantities of virus. These circumstances could increase the probability of human-to-human transmission and may lead to the selection of variant viruses more able to infect humans (Cohen 1997).

Since there is currently no evidence that any such events have occurred (Breman and Henderson, 1998; Cohen, 1997) it does not mean that they could not occur. This argument could not be used to reintroduce a large-scale vaccination with VV. Immunity to othopoxvirus infection may be a beneficial side-effect of the widespread use of VV as a vector for foreign antigens. However, the process of recombinant production may attenuate VV thereby reducing its immunogenicity (Buller *et al.*, 1985). The mechanism of this attenuation is unknown. One possibility is that it is due to impaired DNA replication in some cell types, where a suboptimal level of cellular thymidine kinase may limit the rate of VV DNA synthesis and ultimately the level of expression of VV proteins essential for virus dissemination. It is uncertain what level of immunity to orthopoxvirus infection that vaccination with such viruses may provide. Indeed, a vigorous response to VV may be considered undesirable in recombinants, as immunity may prevent boosting (Belyakov *et al.*, 1999).

The present study has examined the immune response to wt VV and 3 recombinant viruses produced in the VV LIS, a strain with a good safety record (Fenner *et al.*, 1988), that may be acceptable to current licensing authorities. The IgG response, in comparison to the wt virus, was significantly reduced in all recombinants approximately 15-fold. The IgG subclass profiles were typical of the response to live virus

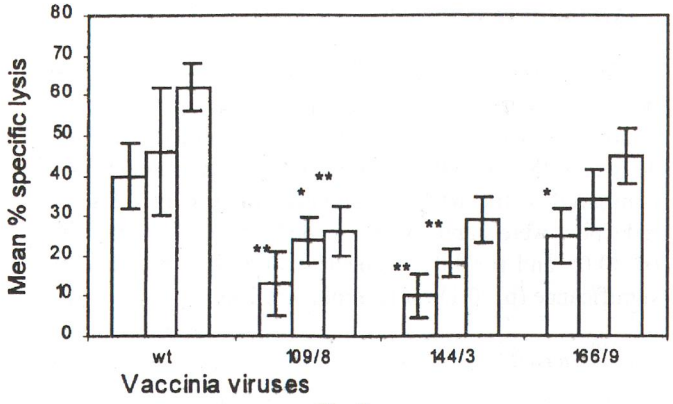

Fig. 3
CTL activity
CTL activity in splenocytes (after secondary stimulation *in vitro*) from mice (N = 5) on day 7 p.s. with $10^{7.7}$ PFU of wt or recombinant VVs. E:T ratios of 12.5:1 (left bar), 25:1 (center bar), 50:1 (right bar). Bar = 95% CI. p <0.05 (*), p <0.01 (**) in comparison to wt VV.

in mice (Coutelier *et al.*, 1987) and were indistinguishable in antisera to wt or recombinant virus. Similar profiles suggest that expression of VEEV antigens did not alter the cytokine environment in which the immune response to VV occurred, and so did not bias the response towards one that may be less protective.

In recombinant virus-vaccinated mice, there was also a significant reduction in IgG specific for the product of the B5R gene, gp42. This EEVV outer membrane glycoprotein is expressed in the LIS strain (Takahashi-Nishimaki *et al.*, 1991) and is associated with virulence (Englestad and Smith, 1993). The antibody to p42 is associated with protection against infection and may neutralize VV (Galmiche *et al.*, 1999).

In functional assays we examined mouse antisera to wt (LIS) and recombinant viruses for neutralizing antibodies to both EEVV and IMVV. EEVV is the form associated with virus dissemination in cell cultures (Blasco and Moss, 1991, 1992; Payne, 1980) and dissemination of virus in the infected host (Payne, 1980; T. Lescott, unpublished data). Antibody to EEVV both neutralizes the virus and protects against infection (Payne, 1980). There were no neutralizing antibodies to EEVV in any of the sera tested, but there was a significant reduction in neutralizing antibodies to IMVV, consistent with the lower IgG levels found in the antisera to recombinant VV.

There was a significant reduction in CTL activity in the spleens of mice vaccinated with recombinant as compared to wt VV at day 7 p.s. The assay was performed after secondary stimulation of spleenocytes *in vitro* and probably represents MHC-class 1-restricted activity. MHC class 1-restricted CTLs may be long-lived after VV vaccination, and are believed to be associated with protection against and recovery from disease (Boulter, 1969; Demkowicz, 1996).

To evaluate the efficacy of wt vs. recombinant VV vaccination in protection against orthopoxvirus infection in the laboratory would require the accurate estimation of the relative lethality (median lethal dose, LD_{50}) or infectivity of selected orthopoxviruses for wt- or recombinant virus-vaccinated, and unvaccinated animals. Both peripheral and airborne routes of challenge would be required. The large number of animals required for these experiments could not be justified. Furthermore such experiments would suffer from the criticism that definitive answers to such questions could only be obtained by studying natural infection, in vaccinated and unvaccinated human populations.

Our data from both antibody binding and functional assays, relevant to protective immunity, indicate that vaccination with recombinant- as compared to wt-VV elicits a reduced immune response. In order to achieve the levels of immunity traditionally associated with protection against orthopoxvirus infection (Fenner *et al.*, 1988) it will be necessary to vaccinate with wt VV. The effect of immunity induced by immunization with VV recombinants on intercurrent orthopoxvirus infection, e.g., MPV remains uncertain. However, should it again become necessary to control an outbreak of human orthopoxvirus infection the vaccination with wt VV seems essential.

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