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Differential efficacy of vaccinia virus envelope proteins administered by DNA immunisation in protection of BALB/c mice from a lethal intranasal poxvirus challenge

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Abstract

DNA vaccines might offer an alternative to the live smallpox vaccine in providing protective efficacy in an orthopoxvirus (OPV) lethal respiratory challenge model. BALB/c mice were immunised with DNA vaccines coding for 10 different single vaccinia virus (VACV) membrane proteins. After an intranasal challenge with the VACV IHD strain, three gene candidates B5R, A33R and A27L produced ≥66% survival. The B5R DNA vaccine consistently produced 100% protection and exhibited greatest efficacy after three 50 μg intramuscular doses in this model. Sero-conversion to these vaccines was often inconsistent, implying that antibody itself was not a correlate of protection. The B5R DNA vaccine induced a strong and consistent gamma interferon (IFNγ) response in BALB/c mice given a single DNA vaccine dose. Strong IFNγ responses were also measured in pTB5R immunised C57BL6 mice deficient for MHC class I molecules, suggesting that the memory response was mediated by a $CD4^+$ T cell population.

Keywords: DNA immunisation; B5R; Gamma interferon

1. Introduction

The World Health Organisation (WHO) announced the eradication of smallpox in 1980 and subsequently recommended that global vaccination should cease [1]. Routine vaccination against smallpox has not been practised for over 20 years, leaving the population of the world increasingly vulnerable. Today the majority of children and adults are not vaccinated against smallpox, and the consequences of a re-emergence of the disease, by whatever means, would be far reaching without effective public health interventions [2,3].

Infection with variola virus (VARV, the causative agent of smallpox) can be initiated by just a few virions [4] and patients remain asymptomatic for between 7 and 17 days. The virus is transmitted principally by the aerosol route and can produce 100% morbidity and up to 40% mortality [1]. Clinical signs appear at the onset of a secondary viremia with fever, followed by headache, backache and the development of a distinctive rash [5,6]. Laboratory diagnosis can take several days and may require biosafety level IV facilities.

The live smallpox vaccine (vaccinia virus) is administered by scarification, and results in swelling, irritation and discomfort at the site of inoculation. The resulting lesion sheds live virus until scabbing occurs at 7–10 days post-inoculation. Vaccination with the Lister (Elstree) or Wyeth (New York City Board of Health) strains of VACV was the method promoted by the WHO during the smallpox eradication campaign in the 1960s and 1970s [1].

VACV and VARV both belong to the genus Orthpoxvirus, that also includes monkeypox virus (MPXV). Monkeypox is a very similar disease to smallpox and has a 1–14% case-fatality rate and routinely causes severe infections in young children. MPXV is an endemic/zoonotic OPV of sub-Saharan Africa [7]. Infections are frequently transmitted to man via infected bushmeat, but person-to-person contact infection can occur, usually between close family

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Table 1
Information relating function and known antigenic properties of the VACV proteins selected for study

VACV gene	Particle type	Protein	Requirement for plaque formation	Neutralising antibody	Protection (%)	Description of gene product
A13L	IMV	p8	Essential	No		[22]
A27L	IMV	p14	Essential	Yes	10 [18]	[23]
D8L	IMV	p32	Essential	Yes		[24]
H3L	IMV	p35	Essential	No		[25]
L1R	IMV	p25	Essential	Yes	80 [17]	[26]
A33R	EEV	gp23–28 (type II glycoprotein	Essential	No	100 [16], 70 [17]	[27]
A34R	EEV	gp22–24 (type II glycoprotein	Essential	No	18 [16]	[28]
A56R	EEV	gp86 (type I glycoprotein)	Non-essential	No		[29]
B5R	EEV	gp42 (type I glycoprotein)	Essential	Yes	82 [16], 40 [18]	[30]
A36R	IEV	gp43–50 (type Ib glycoprotein)	Essential	No	50 [16]	[31]

members [8]. MPXV can be controlled with the smallpox vaccine. A recent outbreak of MPXV in the USA highlights the vulnerability of an unvaccinated population against this poxvirus [9].

Primary vaccination with the current smallpox vaccine is also associated with rare but life-threatening complications [1]. Generalised vaccinia rash occurs as a result of a systemic infection with transient viremia. Prognosis for this condition is usually good. Eczema vaccinatum and progressive vaccinia are life-threatening complications that result from the pre-existing conditions of eczema or immunodeficiency, respectively. Eczema and immunodeficicency in the world population today are more common than 30 years ago and so the risks associated with the current live smallpox vaccine are significantly increased. Live vaccine also poses the threat of accidential infection of the eye, perineum and vulva [1] and the potential for infecting close-contacts such as partners, friends and family.

Subunit vaccines can stimulate strong protective immune responses with reduced side effects relative to complex live or inactivated vaccines. Subunit vaccines have been previously adopted as safe and effective countermeasures for the control of significant viral infections of man such as hepatitis B and influenza [10,11].

The mechanism of protection from VARV infection is poorly understood. Studies with other viruses have identified virion surface antigens as key vaccine components [12,11] perhaps because they have important functions for the virus in cell adsorption, cell entry or virion egress. The identification and characterisation of analogous components of VACV (Table 1) provides the opportunity to elucidate their importance in immunity by targeting individual proteins. All OPVs produce two types of virus particle with distinct surfaces. The intracellular mature virus (IMV) is retained within the infected cell whilst the extracellular (EEV) form of virus is actively secreted from cells and contributes to the efficient dissemination of virus in vitro [13] and in vivo [14]. The EEV membrane contains at least four viral proteins (Table 1). In addition, cellular membrane proteins CD46, CD55, CD59, CD71, CD81 and major histocompatibility complex (MHC) class I antigens have been detected in purified EEV membranes [15]. The surface of the IMV membrane contains a different set of proteins (Table 1). Recent published studies have indicated that subunit protein and DNA vaccines derived from two EEV proteins B5R and A33R are protective [16].

Our studies compared DNA vaccines for 10 different EEV, IMV and one intracellular enveloped virus (IEV) protein to elucidate the efficacy of individual VACV membrane proteins. We show that the EEV proteins B5R and A33R, as well as one IMV protein, A27L, were the most effective in protecting BALB/c mice from a VACV challenge in this model. The B5R DNA vaccine produced 100% protection and an immediate and strong IFNγ response but did not produce reliable antibody responses, suggesting that the main element of protection offered by this vaccine is by cell-mediated immunity.

2. Materials and methods

2.1. Production of DNA vaccines

The VACV genes used in this study included envelope proteins from IMV, EEV and IEV (Table 1). These genes were PCR amplified from VACV IHD-J DNA using Taq DNA polymerase with the primer pairs listed in Table 2, which contained initiation codons and stop codons in the forward and reverse primers, respectively. Amplicons were cloned directly into the mammalian expression vector pTarget (Promega). The gene inserts of individual clones were orientated by restriction digestion and their DNA sequenced (Oswel) to establish an intact and representative open reading frame. Plasmid DNA was prepared in bulk from suitable clones using the Qiagen endotoxin-free mega prep kit and DNA was resuspended in sterile PBS. DNA concentrations were determined by UV spectroscopy.

2.2. Administration of vaccine

Six-week-old female BALB/c or C57BL6 mice were each immunised with 50 μg of endotoxin-free plasmid DNA mixture containing 25% bupivicaine hydrochloride

Table 2
Primers used to amplify VACV IHD-J genes by PCR

VACV gene	Forward primer (reverse primer)	Amplicon size (bp)	Plasmid DNA vaccine
A13L	5′-GGAATTCGGATCCTAAAATGATTGGTATTCTTTTGTT-3′ 5′-CCCGGGCTCGAGTTATACAGAAGATTTAACTAGA-3′	242	pTA13L
A27L	5′-GATCGAATTCTAAAATGGACGGAACTCTTTTC-3′ 5′-AAGCTTCTCGAGTTACTCATATGGACGCCGT-3′	359	pTA27L
D8L	5′-GATCGAATTCAAAATGCCGCAACAACTATCTC-3′ 5′-CCCGGGCTCGAGCTAGTTTTGTTTTTCTCGCG-3′	940	pTD8L
H3L	5′-CCGATTTTAGTAATATGGAATAGTGTTAGA-3′ 5′-ACTAAATGGCGGCGGTGAA-3′	1004	pTH3L
L1R	5′-TATTTAAATGGGTGCCGCAGC-3′ 5′-TTTCTAGTTTTGCATATCCGTGGTA-3′	779	pTL1R
A33R	5′-GATCGAATTCTAAAATGATGACACCAGAAAACG-3′ 5′-AAGCTTCTCGAGTTAGTTCATTGTTTTAACACAAA-3′	584	pTA33R
A34R	5′-GATCGAATTCTAAAATGAAATCGCTTAATAGAC-3′ 5′-AAGCTTCTCGAGTCACTTGTAGAATTTTTTAACAC-3′	533	pTA34R
A56R	5′-CCCGGGATCCTAAAATGACACGATTACCAATACTTTTG-3′ 5′-CGGGCTCGAGTTAGACTTTGTTCTCTGTTTTG-3′	975	pTA56R
B5R	5′-CCGAGTCGACAAAATGAAAACGATTCCCGTTG-3′ 5′-CCCGGGCTCGAGTTACGGTAGCAATTTATGGAAC-3′	980	pTB5R
A36R	5′-GATCGAATTCTAAAATGATGCTGGTACCTCTTATC-3′ 5′-AAGCTTCTCGAGTTACACCAATGATACGACC-3′	692	pTA36R
L1R	5′-TATTTAAATGGGTGCCGCAGC-3′ 5′-TTTTCAGTTTTGCATATCCGTGGTA-3′	779	pTL1R

PCR products were cloned into pTarget and were DNA sequenced. Initiation and stop codons are shown underlined for the forward and reverse primers, respectively.

(Antigen Pharmaceuticals Ltd. Co., Tipperary, Ire) in PBS. Vaccine was applied in a total volume of 100 μl per mouse as two 25 μl intramuscular doses at two points on each hind limb. Vaccines were administered at 3-week intervals and blood samples were taken 1-week post-immunisation.

2.3. Virus production

Vaccinia virus IHD (ATCC VR156) was grown in RK13 cells in Dulbecco's MEM supplemented with 2% foetal bovine serum (FBS) 3 mM glutamine and 100 units/ml penicillin and streptomycin. IMV and EEV was prepared by infecting RK13 cell monolayers with VACV IHD at a multiplicity of infection (MOI) of 10 for 1 h, washing the cell sheet with PBS and adding fresh medium. After 24 h, EEV contained in culture medium was collected, centrifuged at 3000 × *g* for 5 min to pellet debris and then again at 80,000 × *g* for 60 min to pellet EEV. IMV was harvested from the infected RK13 cells at 48 h post-infection by layering the contents of Dounce homogenised infected cells onto a 36% (w/v) sucrose cushion and ultra-centrifuging at 80,000 × *g* for 80 min. Virus pellets were resuspended in PBS, titred and stored at −80 °C.

Purified virus or cell homogenates were heat inactivated at 60 °C for 2 h in 100 μl aliqouts on a PCR thermal block. Ten percent of inactivated virus was put into culture for 7 days to monitor for viral sterility.

2.4. Mouse challenges

After four vaccine doses, given at intervals of 3 weeks, mice were intranasally challenged with 100 MLD_{50} (equivalent to 1.0×10^7 pfu per mouse) of VACV IHD administered as 10 μl to a single nares. Challenge took place 3 weeks after the final immunisation. Daily weights and clinical signs of disease were taken for groups of five or six mice. Humane endpoints for this model have been previously established as either 30% body weight loss, or acute clinical signs such as blindness or severe breathing difficulties. Individual mice were assessed daily for signs of disease and these were assigned numerical scores from 0 (normal), 1 (slightly ruffled), 2 (ruffled), 3 (hunched), 4 (oedema, respiratory distress) to 5 (death or humane endpoint). The sum for each group was expressed as a percentage plotted against time (see Fig. 2), where 100% represents no survivors in a group. Groups with scores below 33.3% exhibited mild clinical signs (protection equivalent to mice scarified with live smallpox vaccine), those between 33.3 and 66.7% exhibited significant clinical signs (some protection) and groups with scores above 66.7% had severe and fatal clinical signs of disease (little or no protection).

2.5. Measuring anti-VACV antibodies

VACV infected cell extract (VICE) antigen was generated from Dounce homogenised VACV IHD infected RK13 cells in PBS. Cell homogenates were centrifuged at 300 × *g* for 10 min to pellet cell nuclei, supernates were dispensed into 1ml aliquots and stored at −20 °C. Ninety-six-well microtitre plates (Immulon-2 HB, Thermolabsystems) were coated overnight at 4 °C with mock-infected or IHD infected RK13 cell extracts in carbonate–bicarbonate buffer (Sigma). Excess binding capacity was adsorbed with Blotto (5% dried milk powder in PBS) for 2 h at 37 °C, and mouse primary antibodies and goat anti-mouse Ig G HRP at 1/1000 (Biorad) were applied for 1 h at 37 °C. Plates were washed three times with PBS supplemented with 0.05% Tween-20. Antigen was coated onto ELISA plates at twice the antigen concentration that produced a maximum optical density (OD) with VACV Lister antisera. Experimental mouse sera were diluted at 1/50 in duplicate wells and were considered positive if they had an OD that was twice the OD obtained for a normal mouse serum. ABTS reagent (Sigma) was used to quantitate antibody binding.

2.6. Measuring IFNγ and IL-5

Female BALB/c mice were immunised as described. Two weeks after immunisation mice were culled and spleens removed and dispersed to single cells. Spleen cells (3×10^5) were cultured in 96-well round-bottomed microtitre plates in 200 μl volumes of RPMI 1640 medium supplemented with 10% FBS, 3 mM glutamine, 20 μM 2-mercaptoethanol and 50 μg/ml gentamycin, in the presence of 5 μg/ml concanavalin-A (ConA) or 100 μg/ml heat-inactivated VICE or extracellular envelope virus (EEV). After 72 h of culture, supernates were removed and stored at −80 °C. Secreted cytokines were measured by ELISA using pre-standardised Pharmingen OPT EIA kits. IFNγ was measured as an indication of a T cell type 1 (T_{c1}) cytokine bias and IL-5 as an indication of a T_{c2} cytokine bias.

3. Results

3.1. Identifying protective membrane protein antigens

We developed a BALB/c mouse challenge model to evaluate the performance of different VACV envelope proteins expressed via a DNA vaccine vector. Ten membrane protein genes (Table 3) were cloned into the mammalian plasmid expression vector pTarget. Five IMV, one IEV and four EEV genes were screened for their ability to protect groups of female BALB/c mice from an intranasal lethal challenge with VACV IHD.

Animals receiving DNA vaccines with the D8L, A33R, A56R and B5R genes were frequently sero-positive against VACV antigen as analysed by ELISA (Table 2). However, the production of anti-VACV reactivity was generally modest, inconsistent for recipients of a given vaccine, and required a minimum of two DNA immunisations (data not shown).

Mice were given an intranasal challenge with 100 MLD_{50} of VACV IHD 3 weeks after four vaccine doses, given at intervals of 3 weeks. Eighty percent of mice receiving pTA27L, 50% receiving pTD8L and 33% receiving the pH3L DNA vaccines were protected from this challenge (Table 2 and Fig. 1a). No mice survived this challenge after receiving the pTL1R or the pTA13L DNA vaccines. Time to death was shorter for the pTA13L group than for the pTarget control group. Hundred percent survival was achieved following challenge by scarification with live Lister strain. The A27L DNA vaccine was the only IMV membrane antigen offering a good level of protection.

The EEV and IEV antigens also included some promising candidates. All mice receiving the pTB5R DNA vaccine

Table 3
Summary illustrating the performance of the 10 DNA vaccines in BALB/c female mice challenged with a lethal intranasal challenge dose of VACV IHD

Particle type	DNA vaccine gene insert	Survivors after challenge (%)	Mean lowest body weight (%)	VACV antibody
IMV	A13L	0 (0/5)	<70	0/5
	A27L	80 (4/5)	85.0	0/5
	D8L	50 (3/6)	73.4	4/6
	H3L	33 (2/6)	73.1	0/6
	L1R	0 (0/6)	<70	0/6
EEV	A33R	67 (4/6)	79.7	4/6
	A34R	0 (0/5)	<70	0/5
	A56R	50 (3/6)	77.0	5/6
	B5R	100 (5/5)	93.7	4/5
IEV	A36R	60 (3/5)	80.3	0/5
Controls	Lister	100 (4/4)	97.7	4/4
	pTarget	0 (0/11)	<70	0/11
	PBS	0 (0/11)	<70	0/11

The presence of VACV antibody was measured by ELISA using VICE antigen. Body weight losses below 30% were not exceeded.

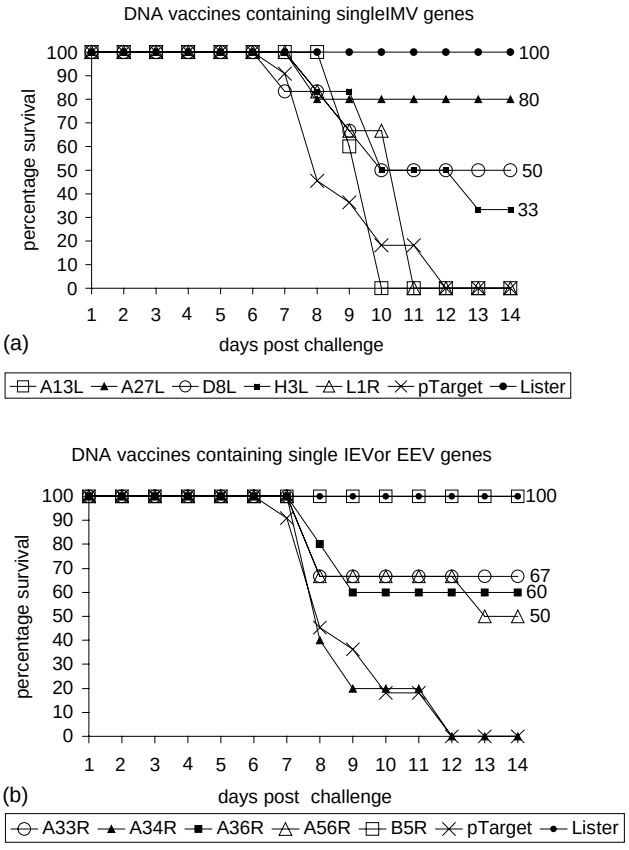

Fig. 1. Survival of groups of BALB/c mice immunised with four doses of DNA vaccine for IMV (a) or EEV/IEV (b) antigens and intranasally challenged with 100 MLD_{50} of VACV IHD. Animals were weighed daily from day 0 (not shown) and were culled if body weight dropped below 30% or if their clinical signs were very severe.

and 66% of mice vaccinated with pTA33R were protected. Sixty percent of pTA36R and 50% of pTA56R DNA vaccinated mice had a body weight loss of <30%, but these two groups exhibited severe signs of disease post-challenge and occasionally relapsed during the recovery phase (data not shown). This indicated that the pTA36R and pTA56R vaccines produced incomplete protection. None of the mice receiving either pTA34R DNA vaccine, PBS or the pTarget empty expression vector survived the challenge. The pTB5R DNA vaccine was the only subunit vaccine that provided 100% protection against a large 100 MLD_{50} VACV intranasal challenge.

3.2. The efficacy of the B5R, A27L and A33R DNA vaccines

Percentage weight loss was considered to be the best objective clinical measurement of vaccine performance against challenge. Final mean percentage weight loss for each group was calculated by taking the minimum weight for each animal over the 14-day challenge, and expressing this as a percentage of the animal's weight immediately prior to challenge. These values were then averaged for the group (Table 3). The pTB5R vaccine produced the best DNA vaccine score with a final mean weight loss of just 7.3%, followed by pTA27L 15% and pTA33R with 20.3% (Table 3). To evaluate how weight loss and signs of disease correlated a scoring scheme was devised that reflected severity. Individual animals were scored daily from zero to five and these scores were summed and plotted as histograms alongside the daily mean percentage weight loss for the group. Data from the empty vector and our three best performing vaccines are shown in Fig. 2. By day 10 the pTarget control group had no survivors and had a maximum disease score and no further daily mean body weight measurement (Fig. 2a). Both the pTA33R (Fig. 2b) and pTA27L (Fig. 2c) DNA vaccines had steep drops in daily body weight up to day 7 and then began to recover. The pTB5R DNA vaccine (Fig. 2d) had the lowest disease score and a shallow weight loss profile relative to the control or the two other vaccines. The rate of recovery as measured by disease signs for the pTB5R DNA vaccine after 7 days post-challenge was much improved compared with pTA33R and pTA27L. This was not apparent from the mean weight loss curve as the pTA33R and pTA27L DNA vaccines did not confer 100% protection (Table 3), and so the data represents survivors only. Final mean percentage weight loss (Table 3) and daily disease scores (Fig. 2) included all mice whether or not they survived. The order of efficacy for the three best DNA vaccine candidates as measured by survival, disease score and weight loss was B5R > A27L > A33R.

3.3. The effect of pTB5R dosing on protection

The effect of multiple pTB5R DNA vaccine dosing was measured to establish the relative magnitude of protection after two, three or four doses of vaccine. All recipients of these pTB5R dose regimes survived the challenge. Fig. 3 shows the effect of dose on protection for a period from 0 to 8 days post-challenge as measured by daily mean percentage weight loss. Up to day 4 post-challenge, a two dose regime was similar to the four dose regime as judged by change in body weight. However, from day 5 onwards the protection was less apparent for the two dose group. Over a 14-day period post-challenge mice receiving three doses of pTB5R DNA vaccine had the smallest percentage mean minimum weight loss 8.0%, compared with 11.0% for the four dose and 17.2% for the two dose regimes.

3.4. Antigen-specific T cell recall responses in pTB5R vaccinated mice

We had observed that antibody was produced by most but not all recipients of the pTB5R DNA vaccine (Table 3). The absence of antibody in mice that were otherwise protected suggests that pTB5R was also capable of inducing a cell-mediated immune response which could be the

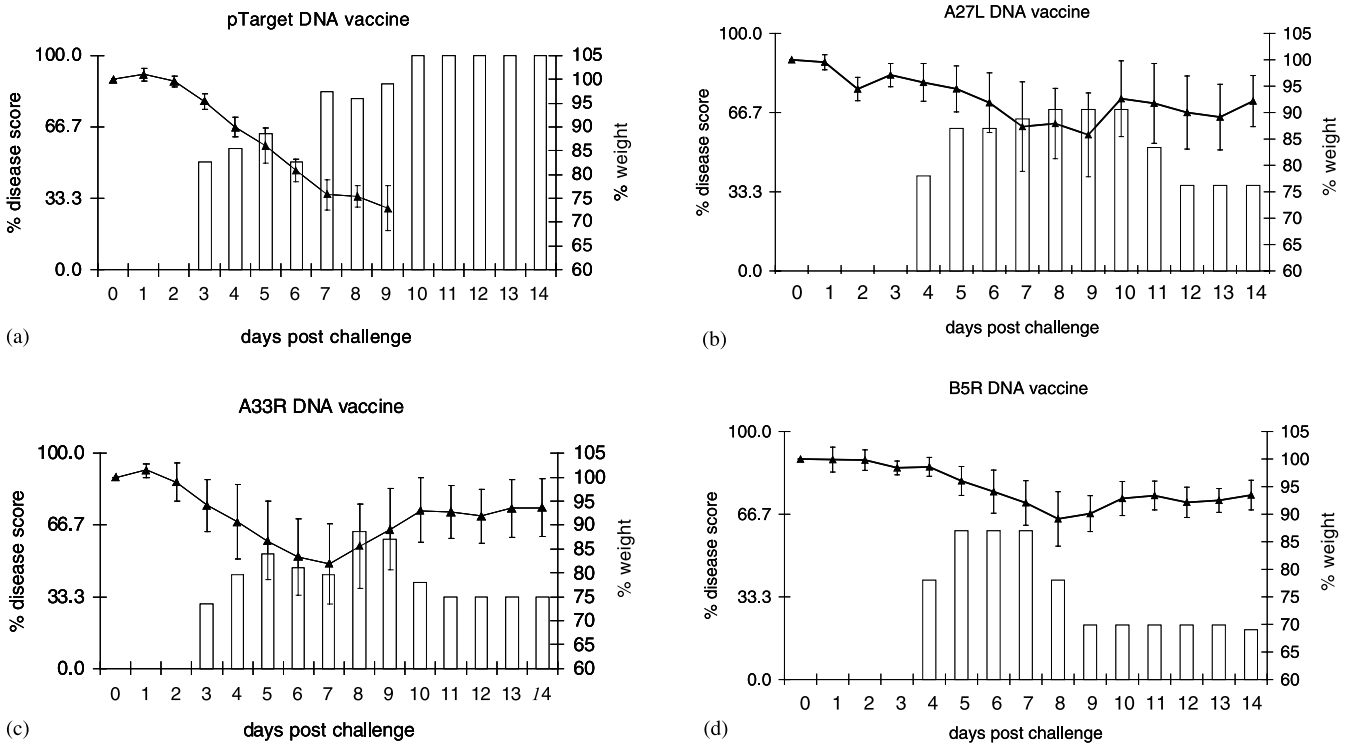

Fig. 2. Combination charts displaying the course of disease in DNA vaccinated groups of BALB/c mice after challenge with 100 MLD_{50} of VACV IHD. The charts display the relationship between percent disease score (shown as columns) and percent mean body weight loss (line) with time post-challenge.

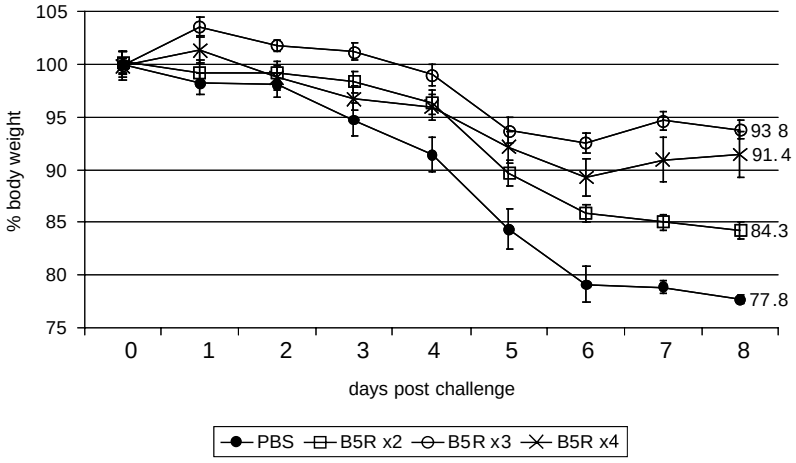

Fig. 3. Effect of vaccine dose on protection from a lethal intranasal VACV challenge. Percentage change in daily mean body weight between days 0 and 8 in mice immunised with two, three or four doses of pTB5R DNA vaccine. Values are shown adjacent to the final data points for all DNA dose regimes on day 8.

basis of protection. To study this we measured IFNγ and IL-5 responses of EEV-stimulated T cells from vaccinated mice as an indication of a type 1 (inflammatory) or type 2 (anti-inflammatory) cytokine bias. BALB/c mice were immunised once, twice, three or four times with 50 μg doses of pTB5R and then spleen cells were harvested and stimulated in vitro for 3 days with or without inactivated purified EEV, a rich source of the B5R protein. IFNγ and IL-5 in the culture supernatants were measured using a quantitative ELISA. No IL-5 production was observed in any pTB5R vaccine group (data not shown). However, strong EEV-induced IFNγ responses were measured in groups of mice given one dose of vaccine with moderate and variable responses for groups given two, three and four doses (Fig. 4). Whilst the mean value in the other dose regimes was lower than for one vaccine dose, individual animals did produce up to 2000 pg/ml of IFNγ in all dose regimes. The priming dose gave the greatest and most consistent cytokine recall.

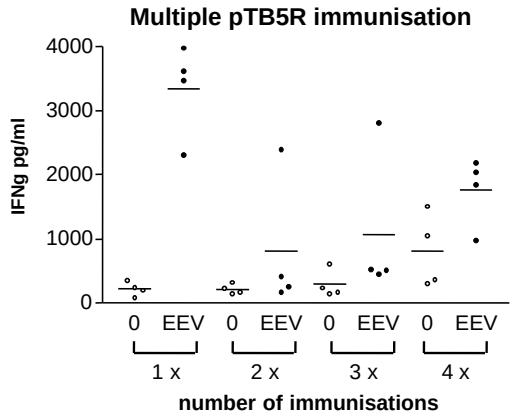

Fig. 4. Effect of dosing with pTB5R to generate a T_{c1}-mediated memory immune response as measured by the production of IFNγ in antigen stimulated spleen cells. Groups of four BALB/c mice were immunised once, twice, three or four times at 3-week intervals with 50 μg of plasmid DNA in 0.25% bupivicaine. Two weeks after each immunisation spleen cells were harvested and grown in vitro in the presence of 100 μg/ml of heat-inactivated mock (0) or VACV IHD EEV. After 72 h culture, supernatants were stored at −20 °C, and then samples were collectively assayed by ELISA using the Pharmingen OPT EIA kits. Standard curves were plotted using recombinant cytokine to enable accurate cytokine quantification.

We vaccinated C57BL6 mice with pTB5R to further determine the cellular origin of the IFNγ (Fig. 5). Unimmunised C57BL6 mice did not produce any IFNγ recall with VICE, except that one out of seven mice did respond to EEV. pTB5R immunised C57BL6 mice produced variable IFNγ responses, in a pattern resembling that for BALB/c

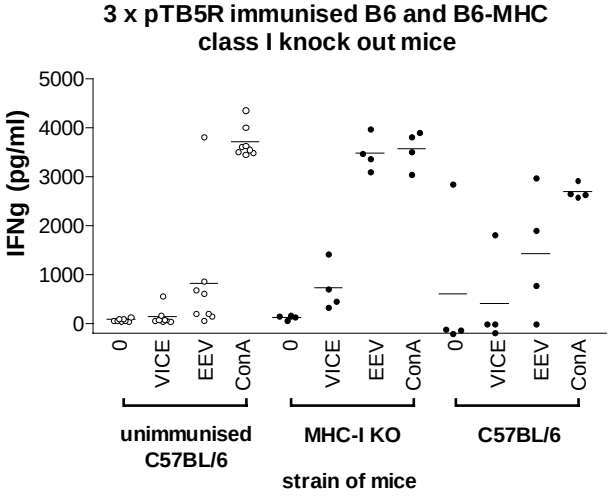

Fig. 5. Monitoring the IFNγ in vitro recall response to VICE or EEV antigens in C57BL/6 or MHC-I knockout mice immunised with pTB5R. Mice were either unimmunised (seven) or immunised (four) with three 50 μg doses of pTB5R at 3-week intervals. Two weeks after the final immunisation spleen cells were harvested and grown in vitro in the presence of 100 μg/ml of heat-inactivated mock (0), heat-inactivated VICE, heat-inactivated EEV antigen or 5 μg/ml ConA. After 72 h culture supernatants were treated as described in Fig. 4.

immunised mice (Fig. 4). One of the four DNA immunised C57BL6 mice also produced a strong IFNγ response (2000 pg/ml) against VICE antigen (Fig. 5). Interestingly, all four pTB5R immunised C57BL6 MHCI knockout transgenic mice produced strong antigen-specific recall responses (>3000 pg/ml) in response to EEV antigen that was equivalent to the response obtained with the polyclonal mitogen ConA. Reduced memory responses were also measured in VICE antigen stimulated C57BL6 MHCI spleen cells that perhaps reflects the lower concentration of the B5R antigen in this preparation.

4. Discussion

A DNA vaccine based on the VACV B5R gene is shown here to provide 100% survival at 14 days post-challenge in a mouse model that attempts to mimic aspects of the natural route of smallpox infection in man. Other envelope protein genes had different abilities to protect mice against a lethal intranasal challenge with VACV, as vaccines based on the A27L, A33R, A36R, A56R and D8L genes all provided greater than 50% survival at 14 days post-challenge (Fig. 1). Three envelope protein genes, A34R, A13L and L1R did not provide any protection against challenge in this study. To date this is the most extensive published survey of individual VACV genes in a single lethal challenge model. The challenge model represents the route of natural infection with smallpox more closely than for other routes (i.e. intra-peritoneal or cardiac infusion), because the challenge virus infects the host via the mucosal surfaces of the respiratory tract before it travels to other tissues and organs. The model therefore maximises the relevance to smallpox airborne infection of the immune mechanisms of action elicited by the individual vaccine candidates.

Our studies and those of Galmiche et al. [16] used a respiratory challenge model and have produced similar findings, though our study used a 10-fold higher challenge dose of VACV IHD. The survival rates in the study of Galmiche et al. [16] were 100% for A33R, 82% for B5R, 50% for A36R and 18% for A34R. Interestingly, these authors found that the A33R gene, not the B5R gene, provided the most complete protection, but their results also showed that B5R vaccinated mice recovered faster than the A33R mice by weight measurement, suggesting that the B5R vaccine was effective. Our results broadly support the findings of [16], and indicate that A33R and B5R are both effective DNA vaccine candidates. We show that the B5R DNA vaccine invokes a potent IFNγ production in vitro upon exposure to EEV antigen that might be the basis of conveyed protection in vivo.

Another study that used different challenge and immunisation routes produced different results with the same DNA vaccine candidates used in our study. Hooper et al. [17,18] looked at the individual and the combined effects of A27L, A33R, L1R and B5R by gene-gun immunisation in a

challenge model that used 5×10^8 pfu VACV (WR strain) cell lysate (12 LD_{50}) administered by the intraperitoneal route. After three gene-gun immunisations with 0.5–1 μg of DNA, only 10% of A27L, 40% of B5R [18] and 70% of A33R [17] DNA vaccine recipients survived the challenge. The A27L gene product (p14k fusion protein) stimulates neutralising antibody in the context of an infection with live VACV. These authors demonstrated that the A27L DNA vaccine produced neutralising antibody titres of 1/160 to >1/640, but the use of a challenge preparation containing free antigen could compete with challenge virus for antibody binding. This may conceivably have lead to an underestimation of the protective efficacy of the A27L-based vaccine, and indeed for other candidates in their study which induced significant levels of antibody. However, the L1R gene produced 80% protection in the study by Hooper et al. [17], but the L1R gene provided no protection in the study we report here. There is no obvious explanation for this discrepancy, which suggests that some of the differences between the two studies may be the result of potential artefacts such as expression levels from DNA constructs, route of immunisation, the strain of challenge virus and/or route of challenge.

pTB5R, pTA33R, pTA56R and pTD8L were the only DNA vaccines that produced anti-VACV circulating antibody in this study, but sero-conversion was inconsistent and the titres achieved by ELISA with VICE antigen were modest (<1/100) (Table 3). In subsequent experiments, we have found that pTA27L can induce antibody in a small proportion of vaccinees (data not shown). However, the ability of pTA27L to provide 80% protection (Fig. 1) in the absence of any measurable antibody (Table 3) implies that other immune mechanisms are activated by the pTA27L vaccine. This is also true for some animals receiving the pTB5R and pTA33R vaccines.

Consistent sero-conversion was obtained with all four DNA vaccines studied by [17,18] and strong neutralising antibody titres were achieved for A27L and L1R, which implies that gene-gun administration improves the humoral immune response. The gene gun injects DNA efficiently into subcutaneous tissues, where dendritic cells may be expected to encounter the DNA and any antigen that is produced from it. In our own study, and that of [16] immunisation was by intramuscular injection. This places the antigen source in an immunologically different environment to that used by Hooper et al. [17,18] and this may be a significant factor in the differential induction of antibody, and survival post-challenge in the three studies.

As antibody was not a correlate of protection in our study, we then went on to determine if the pTB5R vaccine was stimulating a T_{c1}-type response. We measured strong IFNγ production in in vitro stimulated spleen cells from vaccinated animals, which is indicative of a type 1 T cell (inflammatory) cytokine bias in the response. IFNγ was produced at high levels immediately after a single vaccination. Additional immunisations did not appear to enhance IFNγ production but a gradual increase in IFNγ upon boosting was observed and might represent the tail of rapid but decaying large secondary responses. Further studies are necessary to illuminate the dynamics of IFNγ production in BALB/c mice and reveal how primary and secondary immune responses impact on this important cytokine's synthesis.

The functions of IFNγ include stimulating expression of MHC class I and II and activation of macrophages and NK cells, and it plays a major role in enhancing the adaptive immune response. We then used a different mouse genetic background with the C57BL6 strain to determine whether CD4 or CD8 T cells were the predominant source of IFNγ (Fig. 5). C57BL6 mice produced variable antigen-specific memory responses after three vaccine doses similar to vaccinated BALB/c mice (Fig. 4). However, the C57BL6 MHC class I knockout mice, which lack most CD8+ cells, consistently produced high levels of IFNγ in response to in vitro recall with EEV antigen, suggesting that CD4+ T cells were the main source of IFNγ in these mice. Although equal numbers of spleen cells were used from each mouse strain, the absence of CD8+ cells in C57BL6 MHC class I knockout spleens must bias the cell composition in favour of CD4+ cells. In addition, our use of inactivated virus antigen to measure memory responses excluded the opportunity to measure significant CD8 responses. Only quantitative fluorescent staining of CD4 and CD8 cells for IFNγ in these mice will reveal the full nature of the IFNγ dynamic with these mice strains.

Poxviruses have evolved strategies to modulate the effects of IFNγ production. VACV produces a soluble IFNγ receptor molecule (B8R gene) that serves to bind and sequester soluble IFNγ, and other poxviruses have been shown to encode proteins that interact with IFNγ or its receptors [19,20]. The fact that poxviruses specifically modulate this cytokine suggests that IFNγ might have an important role in developing a protective immune response. Recent studies with the live smallpox vaccine indicate that IFNγ producing human T cell responses are a consistent marker after vaccination [21]. Our data suggest that IFNγ production post-vaccination may prove to be an important correlate of immunity for any eventual subunit or DNA vaccine against OPV infection.

Further work is required to understand the mechanisms of protection that are induced by the pTB5R and other DNA vaccines, and to establish definitive correlates of immunity. Our work and that of others indicate that antibody is not the only source of protection. The DNA vaccines examined here do confer some level of cell-mediated immunity, and understanding this immune response will be important if a safe subunit or DNA alternative to live smallpox vaccine is to be developed.

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