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Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus

Brief Report

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Summary. In vivo transfection by intramuscular injection with plasmids expressing the immunogenic proteins of microbial pathogens has considerable potential as a vaccination strategy against many pathogens of both man and animals. Here we report that weanling mice given a single intramuscular injection of 50 μg of a plasmid, pSLE1 expressing the St. Louis encephalitis virus (SLE) prM/E protein under the control of the cytomegalovirus immediate early protein promoter produced SLE-specific antibody and were protected against lethal challenge with the virulent virus. Polynucleotide vaccine technology provides a unique opportunity to produce vaccines against flavivirus diseases of low incidence cheaply and rapidly, and to produce multivalent vaccines such as would be required for immunisation against dengue virus disease.

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Live attenuated virus vaccines are very effective, multiplying in the host to generate a wide range of antigens. These are appropriately processed and presented to the immune system, and may elicit the immune effectors necessary for protection against future exposure to virulent virus. However, live vaccines may prove pathogenic in immunocompromised individuals and may revert to virulence. The latter is of particular concern in RNA viruses which have high mutation rates, especially where attenuation has been achieved by repeated passage and the nature of the attenuating mutations is not known. Where the protective antigens of a pathogen have been identified, subunit vaccines offer the possibility of a defined product free from reactogenicity. However subunit vaccines are expensive to produce and often require repeated injections with potent adjuvants. They are also poor at generating class 1 MHC-mediated

cytotoxic T cells (CTL) which may play an important part in protection against virus disease.

The original demonstration by Wolff and colleagues [1] that there may be long-term persistence and foreign gene expression after intramuscular injection of plasmid DNA suggested that in vivo transfection could be a promising means of immunisation. Vaccination with polynucleotide vaccines has been shown to protect against infection with influenza A virus [2, 3], rabies virus [4] and lymphocytic choriomeningitis virus [5]. Immune responses have also been elicited by polynucleotide vaccines to hepatitis B virus surface antigen [6] and HIV-1 [7]. Both neutralising antibody and CTL have been demonstrated. Here we report that immunity against infection with the encephalitogenic flavivirus St. Louis encephalitis virus (SLE) may be conferred by intramuscular immunisation of mice with a polynucleotide "naked DNA" vaccine.

Cloning of the SLE virus prM/E sequence into the pGEM vector has been described [8]. Briefly, the DNA encoding the prM/E region of SLE virus (nucleotides 372–2460) was amplified by RT-PCR of viral RNA from infected mouse brain and the gel purified PCR product was cloned into the pGEM-T vector following the manufacturer's instructions. Plasmid vector pMV100 (kindly provided by Dr. G. W. G. Wilkinson of CAMR, Salisbury, UK) was used for naked DNA immunisation experiments. pMV100 is a transient expression vector with the cytomegalovirus (CMV) major IE promoter and terminator [9]. The SLE virus prM/E expression cassette with the initiation and termination codons was excised from the pGEM-T vector on a BamH1 fragment and inserted into the BamH1 site of pMV100 downstream of the promoter to generate the plasmid pSLE1.

The correct transcriptional orientation of the prM/E coding sequence and CMV IE promoter was confirmed by restriction digestion analysis of the plasmid pSLE1 with Sph1. Plasmid DNA was prepared using a Qiagen Tip kit according to the manufacturer's instructions. Purified DNA was estimated by absorbance at 260 nm and stored at −20 °C. In vitro transfection of Vero cells using the Lipofectin method, followed by immunoflourescent staining with mouse polyclonal anti-SLE serum (kindly supplied by Dr. N. Karabatsos of CDC, Atlanta, USA) confirmed the expression of virus-specific proteins (data not shown).

For protection experiments female 3–4 week old Porton TO strain mice were injected intramuscularly into the dorsal anterior tibialis muscle on day 0 with 50 µl PBS containing either 50 µg pSLE1, 50 µg pMV100 (without the SLE coding sequence) or PBS alone. On day 21 some mice were given a further intramuscular injection of 100 µg pSLE1 in 50 µl PBS. Serum samples, diluted in 1 ml of PBS were taken from all mice by tail bleeding on day 42 seven days prior to virus challenge.

The strain of SLE virus used in these studies, MS1–7 (kindly supplied by Dr. E. Gould of the NERC Institute of Virology, Oxford, UK) is not lethal for adult mice after peripheral inoculation. However preliminary experiments showed that ic injection with 50 µl of Liebovitz L15 medium containing 2% foetal calf serum (mock ic), followed approximately 1 h later by sc challenge with MS1–7

(volume 100 µl in L15 medium) was lethal. The time course of this infection was similar to that produced by sc challenge of 3–4 week old mice (first deaths at 7–8 days) and slower than the timecourse following direct ic inoculation of virus (first deaths of 4–5 days; data not shown). These relationships suggested that after mock ic/sc challenge there was peripheral replication of the virus with a subsequent viraemia, followed by spread through the traumatised and inflamed blood-brain barrier to the CNS. This schedule was therefore adopted in preference to direct ic inoculation, as it appears to mimic natural infection and was thought likely to provide greater exposure of the challenge virus to the immune system.

On day 49 all groups of mice were challenged mock ic/sc with $30LD_{50}$ of SLE strain MS1–7 (1000 pfu, estimated by assay in human adenocarcinoma SW13 cells). In order to minimise suffering of the animals, severely ill mice (low mobility, hunched posture and signs of paralysis) were culled before death due to the infection ("humane endpoints"). Surviving mice were followed for 18 days before culling. The results of virus challenge are summarised in Fig. 1.

Significant protection was achieved against SLE virus by a single im injection of 50 µg pSLE1 in comparison to control groups which received either 50 µg plasmid without the SLE coding sequence or PBS alone ($p < 0.05$, $p < 0.01$ respectively). Although significant protection was achieved with the 50 µg + 100 µg pSLE1 given im in comparison to PBS ($p < 0.1$) there appeared to be no advantage in giving two immunisations.

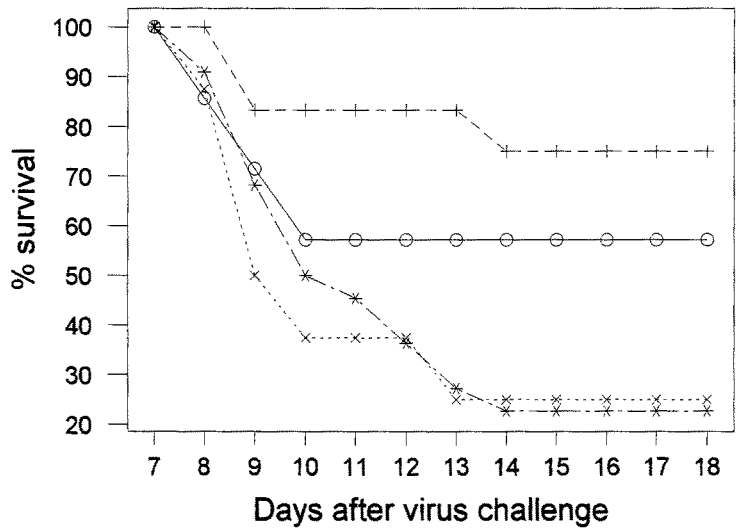

Fig. 1. Survival of mice after virus challenge. Mice were examined daily and humane endpoints were used (see text). *PBS [22], × pMV100 50 µg [8], ○ pSLE1 50 µg + 100 µg [7], + pSLE1 50 µg [12]. Numbers in parenthesis are the number of mice in each group. One mouse which died on day 2 (50 µg vector group) probably due to the trauma of anaesthesia and ic inoculation, was not included in the analysis. 50 µg pSLE1 vs 50 µg pMV100 vector (9/12 vs 2/8) $p < 0.05$, 50 µg pSLE1 vs PBS (9/12 vs 5/22) $p < 0.01$, 50 µg + 100 µg pSLE1 vs PBS (4/7 vs 5/22) $p < 0.1$ (Fisher's exact test). Other comparisons $p > 0.1$

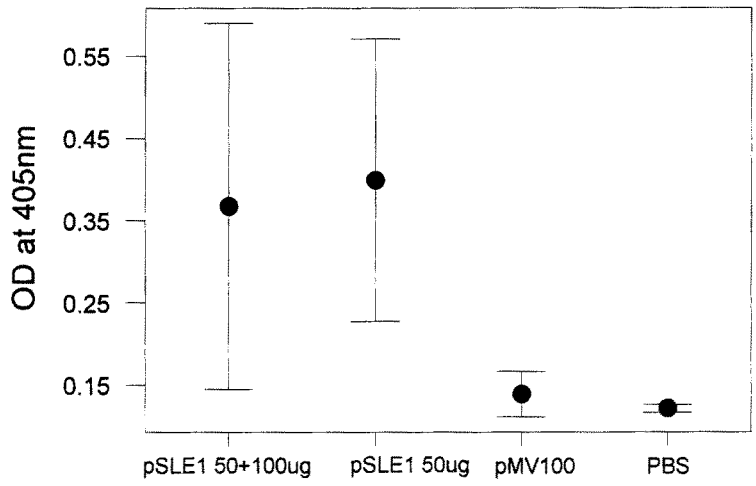

Fig. 2. ELISA results on pre-challenge sera. Error bars show 2 × SEM. In pairwise comparisons using the two-sample T test both pSLE1-immunised groups had significantly higher antibody levels than either the pMV100 vector-immunised or PBS-immunised control groups ($p < 0.01$). Other comparisons were not significant

Post immunisation, pre-challenge serum samples (day 42) were assayed for protein using the BCA method (Pierce, UK) and adjusted to 20 μg/ml total protein. Samples were tested without further dilution in an ELISA assay in which plates were coated with live SLE virus antigen deposited from infected Vero cell culture fluid, or mock antigen prepared from uninfected cell culture fluid. Bound immunoglobulin was detected by the addition of 1/4000 peroxidase-labelled goat anti-mouse IgG (Sigma) followed by tetramethyl benzidine substrate [10] and absorbances were read at 405 nm. There was no significant binding of IgG from any samples to mock antigen (data not shown). The results of binding to SLE-infected and uninfected ELISA antigen are summarised in Fig. 2.

Sera from mice immunised with pMV100 vector or PBS alone gave mean absorbances comparable to reagent controls whereas sera from mice immunised with 50 μg + 100 μg pSLE1 or 50 μg pSLE1 gave mean absorbances as much as two to three-fold higher. In pairwise comparisons of absorbance values using the two-sample T-test there was no significant difference between the two pSLE1-immunised groups and therefore no increase in antibody due to boosting was demonstrated. Both pSLE1-immunised groups had significantly greater antibody reactivity with SLE-infected ELISA antigen than either of the control groups ($p < 0.01$ for either comparison).

In order to confirm the specificity of the antibody produced by pSLE1-immunised mice, western blots were prepared from 12.5% SDS-PAGE gels of denatured control and SLE-infected antigen. These were reacted with pooled tail bleed sera having high (> 0.7) absorbance values from the 50 μg pSLE1 group and pooled negative samples from the plasmid vector group. In order to identify the position of protein prM/E and its cleavage products, blots were also reacted with a rabbit antiserum raised to prM/E expressed by a recombinant baculovirus

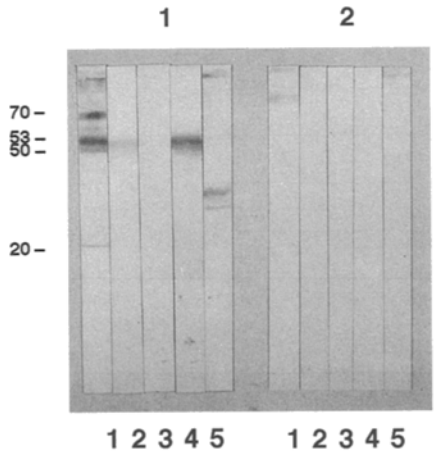

Fig. 3. Western blots of pre-challenge sera. *1* SLE-infected Vero antigen, *2* Vero control antigen. *1* Rabbit anti-prM/E 1/500, *2* pooled ELISA-positive sera from pSLE1-immunised mice, *3* pooled pre-immunisation sera from the 50 μg pSLE1-immunised group, *4* mouse anti-JE serum 1/100, *5* normal rabbit serum 1/100

and purified from infected Sf9 cells (kindly supplied by Dr. E. Gould of the NERC Insitute of Virology, Oxford, UK). Mouse antiserum to the serologically related flavivirus Japanese encephalitis (JE) virus (kindly supplied by Dr. N. Karabatsos of CDC, Atlanta, USA) gave further confirmation of the position of the principle virion surface glycoprotein E.

Rabbit anti prM/E revealed four major bands of MW approximately 20 k, 50 k, 53 k and 70 k (Fig. 3). These have appropriate MWs for and may represent preM, non-glycosylated and glycosylated E, and unprocessed prM/E respectively. This idea is supported by recognition of the 50 k and 53 k bands by mouse polyclonal antiserum to JE virus (which cross reacts with SLE E). Equivalent bands did not stain in blots prepared from mock (uninfected) cell antigen or in SLE-infected antigen blots reacted with normal rabbit serum or negative pooled mouse sera from the plasmid vector-immunised group used at comparable dilutions. However pooled ELISA positive sera from 50 μg pSLE1 immunised mice revealed bands at both the 50 k and 53 k positions suggesting the presence of SLE E protein-specific antibody.

To test for the presence of neutralising antibody, ten-fold dilutions of SLE virus were mixed with an equal volume of selected high absorbance value pre-challenge serum samples (total protein content 20–50 μg/ml) from the 50 μg pSLE1 group and the residual virus estimated by plaque assay on SW13 cells. No virus neutralising activity was detected (data not shown) despite the presence of antibody to gE (some of the samples tested for neutralising antibody were used for Western blotting). Our failure to detect neutralising activity may have been due to the relatively high dilution ($> 1/1000$) of the serum samples used.

In the group of over 68 flaviviruses there are 22 significant human pathogens [11]. The attenuated vaccine produced from 17D yellow fever and related strains has proved one of the most stable and successful attenuated virus vaccines ever developed (Flavivirus vaccines have recently been reviewed in [12–15]). Candidate attenuated vaccine strains for JE and dengue are currently undergoing trials of safety and efficacy but have yet to find wide acceptance for use in man. Two

further flavivirus vaccines are widely available against Japanese encephalitis and Central European tick-borne encephalitis, both of which are inactivated whole virus preparations.

The development of vaccines against all 22 human flavivirus pathogens is not only a daunting task scientifically, it may also be economically non-viable because of the low incidence of disease produced by many flavivirus pathogens (eg. SLE, Murray Valley encephalitis) and their occurrence in impoverished third-world countries. In addition the occurrence of severe life-threatening haemorrhagic disease as a result of sequential infections with different dengue serotypes suggests that simultaneous immunisation against all four dengue serotypes will be a prerequisite for a successful dengue vaccine.

The data presented here show that a polynucleotide "naked DNA" vaccine may immunise against infection and disease caused by a human pathogenic flavivirus, SLE. Specific antibody against SLE virus was elicited by vaccination, although there was no evidence that protection was antibody-mediated. Cell-mediated immunity has been demonstrated after polynucleotide vaccination [5] and may have been responsible for protection in these experiments. However we have no data to confirm this idea.

In addition to low cost and ease of production, polynucleotide vaccines offer a unique solution to two of the major problems of flavivirus vaccinology. Firstly, multiple flavivirus vaccines based upon a single expression plasmid may be possible allowing vaccines against a wide range of flaviviruses to be rapidly and cheaply produced. Secondly it may be possible to design polynucleotide vaccines such that more than one flavivirus could be immunised against in a single vaccination. The genes in such constructs may be expressed coordinately, or individually under the control of separate promoters. Further experiments to examine the feasibility of such approaches should be a high priority in flavivirus vaccine research and development.

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