Immunological basis for protection in a murine model of tick-borne encephalitis by a recombinant adenovirus carrying the gene encoding the NS1 non-structural protein


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Introduction

The flaviviruses form a family of over 60 members, mainly transmitted by mosquito or tick vectors, which are responsible for many of the most important viral diseases of man and animals, including yellow fever, dengue fever, Japanese encephalitis, louping ill and tick-borne encephalitis (reviewed in Chambers et al., 1990).

Successful vaccines against several of these diseases are available, but attempts to develop vaccines against other diseases, especially dengue, have proved difficult, although evidence recently reported to the World Health Organization suggests that new vaccines should soon be available. In addition, the commercial killed vaccines against Japanese encephalitis and tick-borne encephalitis are too expensive for widespread use in many of the populations most in need (reviewed in Stephenson, 1988). Consequently, there has been a significant effort in recent years aimed at employing recombinant DNA technology to produce improved vaccines. Vaccines based solely on the envelope proteins of these viruses may, however, induce an immune enhancement activity, similar to that seen in severe cases of dengue (Halstead, 1982). Antibody-based immune enhancement (ADE) can be readily detected in vitro (Peiris & Porterfield, 1982; Phillpotts et al., 1985) as a rise in virus infectivity, and also in vivo in experimental animals as an increase in levels of viraemia (Halstead, 1979). Furthermore, as the flavivirus E protein appears to be a relatively weak inducer of cytotoxic T cells (CTL) (Bukowski et al., 1989; Rothman et al., 1993), such vaccines may primarily induce the expansion of clones of T helper type 2-like (Th2) cells to the detriment of clones of T helper type 1-like (Th1) cells. Thus, vaccines based on E proteins alone may induce a Th2-based immune response capable of neutralizing virus particles, but be relatively inefficient in inducing a Th1-based immune response which would activate both the complement fixation pathway and the maturation of CTLs.

The non-structural flavivirus proteins can, however, induce good CTL responses in experimental animals, with NS3 being...
the most frequent target identified (Parrish et al., 1991; Rothman et al., 1993; Lobigs et al., 1994). In addition, CD3+ /CD4+ virus-specific T cell lines, mainly NS3- and NS4a-specific, have been developed from human donors (Bukowski et al., 1989; Kurane et al., 1989, 1991). The NS1 protein has been known for some time to bind complement (Cardiff et al., 1970; Falker et al., 1973) and to induce a protective immune response in experimental animals (Schlesinger et al., 1986). In addition, antibodies to it can protect against experimental infections (Schlesinger et al., 1985; Gould et al., 1986). Furthermore, our previous work has demonstrated that defective recombinant adenoviruses expressing only the NS1 protein of tick-borne encephalitis virus (TBEV) induce significant protection in a murine model against infection with the parent virus (Jacobs et al., 1992, 1994).

The safety and efficacy of adenovirus vaccines have been well-documented (Schwartz et al., 1974) and thus vaccines based on deletion mutants of these viruses are good candidates for the next generation of prophylactic agents (reviewed in Rubin & Rourke, 1994; Wilkinson & Borysiewicz, 1995). Indeed, our previous studies (Jacobs et al., 1992; Fooks et al., 1995) and those of many other groups (reviewed in Graham & Prevec, 1992) have demonstrated that these defective recombinant adenoviruses can induce humoral immunity, cell-mediated immunity and provide high levels of protection, at least in experimental animals. Here we describe the components of a protective immune response, with broad specificity, which is induced by a recombinant adenovirus bearing only the NS1 gene of TBEV.

## Methods

### Cells and viruses. TBEV was grown in suckling mice brains as described previously (Stephenson et al., 1984). TBEV isolate ‘205’ was a gift from M. Vorobyova (Tarasevich State Institute, Moscow). Omsk haemorrhagic fever virus (OHFV) was a gift from V. Pogodina (IPVE, Moscow) and Negishi virus was a generous gift from A. Karavanov (IPVE, Moscow); all other virus isolates were from the virus collection of the Chumakov Institute of Poliomyelitis and Viral Encephalitides (IPVE) and have been described elsewhere (Stephenson et al., 1984; Krasilnikov et al., 1984; Dzhivivanyan et al., 1988). The recombinant adenoviruses Rad51 (containing the NS1 gene from TBE virus) and Rad35 (containing the β-galactosidase gene from Escherichia coli) were propagated in 293 cells as described previously (Jacobs et al., 1992; Wilkinson & Akriag, 1992).

### Protection experiments. BALB/c mice (12–14 g, mixed sexes) were inoculated intraperitoneally (i.p.) with 10⁷ p.f.u. of recombinant adenoviruses in 0.2 ml of PBS on day 0 and day 7, and then challenged i.p. with 10–100 LD₅₀ of the desired flavivirus in 0.1 ml of PBS on the 8th day after the last vaccination. Mice were monitored for 3 weeks after virus challenge.

Animals for protection enhancement experiments were immunized subcutaneously (s.c.) on day 0 and day 7 with serial 1 in 3 dilutions (0.2 ml) of concentrated and purified TBE vaccine (lot no. 201) produced by the Chumakov Institute of Poliomyelitis and Viral Encephalitides. Half the animals were given vaccine alone and the other half were also inoculated i.p. with 0.1 ml of PBS containing 10⁷ p.f.u. of Rad51. One week after the final vaccination all animals were challenged with 10–100 LD₅₀ of the Absettarov isolate of TBEV. Animals were monitored for up to 3 weeks and the dilution of vaccine that protects 50% of the animals (PD₅₀) was calculated by the method of Reed & Muench (1938).

### Adoptive transfer protocols. Mice were immunized once with 10⁷ p.f.u. of Rad51 in PBS; spleens were removed after 8 days and homogenized in Medium 199, supplemented with 5% FCS. Cell suspensions were filtered, centrifuged at 400 g for 10 min and the pellet was washed once with Medium 199. Macrophages were obtained by the absorption of 10⁷ spleen cells on 9 cm diameter glass plates for 1.5 h at 37 °C in an atmosphere of 5 % carbon dioxide. The unabsorbed cells were washed off with Medium 199 and the absorbed macrophages were treated with anti-mouse-Thy₁, antiserum and anti-mouse immunoglobulin antiserum (Sigma).

T and B cells were obtained from the unabsorbed spleen cell suspension by fractionation through a syringe containing nylon cotton as described by Henry (1980). Briefly, 10⁷ spleen cells were passed through a 10 ml syringe containing nylon cotton for 45 min at 37 °C. The filtrate, which contained T cells, was treated with anti-mouse immunoglobulin antiserum. B cells were desorbed from the nylon cotton under pressure with 0.85% NaCl and treated with anti-mouse-Thy₁, antiserum. Macrophages, T or B cells (~ 10⁵ of each cell type per mouse-recipient) were injected into the tail vein of mice which were then challenged i.p. 1 h later with 10–100 LD₅₀ of TBEV (strain Absettarov). Aliquots (0.2 ml) of pooled antisera from mice immunized with Rad51 were similarly injected into the tail vein of recipient mice. In some cases, recipients were pre-treated with cyclophosphamide (0.2 mg per mouse) 1 day before the adoptive transfer experiments.

### Assays of antiserum by ELISA. Titres of anti-NS1 IgG and IgM in mouse sera were determined by ELISA using goat anti-mouse IgG and IgM peroxidase conjugate produced by ICN (UK). Concentrated culture fluids of Vero cells infected by Rad51 were used as an antigen. For each data point pooled antisera from not less then three mice were used. Experimental protocols and data analysis were as described by Warness et al. (1994).

### Cytokine bioassays. Tumour necrosis factor (TNF) was measured by the standard cytotoxic test on L1210 cells in the presence of actinomycin D (5 μg/ml), as described by Ruff & Gifford (1981). Total interferon activity was determined using L929 murine fibroblast infected with encephalomyocarditis virus (10⁷ CPE₅₀/ml) in 96-well tissue culture plates as described by Cambell et al. (1975). The results were monitored 24 h after the injection using an inverted microscope and the activity expressed in International Units (IU)/ml.

### Cytokine ELISA assays. IFN-γ was measured using a kit produced by Ciston Biotechnology (Pine Brooks, USA) and the results are expressed in pg IFN-γ/ml of mouse serum.

IL-1 and IL-2 were measured using the Genzyme Biotechnology (Cambridge, UK) kits and the results are expressed in pg/ml mouse serum.

## Results

### Rad51 can protect against infection with other members of the TBE complex

The original demonstration that Rad51 can protect against TBEV infection has now been extended to show its ability to protect against other pathogenic human viruses of the TBE complex.
Table 1. Protective activity of Rad51 in BALB/c mice

<table>
<thead>
<tr>
<th>Vaccination on day:</th>
<th>Virus strain used for challenge</th>
<th>Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>0; 7</td>
<td>TBEV (strain Sophyin)</td>
<td>7/12</td>
</tr>
<tr>
<td>None</td>
<td>TBEV (strain Sophyin)</td>
<td>0/12</td>
</tr>
<tr>
<td>0; 7</td>
<td>TBEV (strain Absettarov)</td>
<td>11/12</td>
</tr>
<tr>
<td>None</td>
<td>TBEV (strain Absettarov)</td>
<td>0/12</td>
</tr>
<tr>
<td>0</td>
<td>TBEV (strain Absettarov)</td>
<td>11/12</td>
</tr>
<tr>
<td>0; 7</td>
<td>TBEV (strain 205)</td>
<td>10/10</td>
</tr>
<tr>
<td>None</td>
<td>TBEV (strain 205)</td>
<td>0/10</td>
</tr>
<tr>
<td>0; 7</td>
<td>TBEV (strain ‘R’)</td>
<td>10/10</td>
</tr>
<tr>
<td>None</td>
<td>TBEV (strain ‘R’)</td>
<td>0/10</td>
</tr>
<tr>
<td>0, 7</td>
<td>Omsk haemorrhagic fever virus</td>
<td>8/10</td>
</tr>
<tr>
<td>None</td>
<td>Omsk haemorrhagic fever virus</td>
<td>0/10</td>
</tr>
<tr>
<td>0, 7</td>
<td>Negishi</td>
<td>2/10</td>
</tr>
<tr>
<td>None</td>
<td>Negishi</td>
<td>0/10</td>
</tr>
<tr>
<td>None</td>
<td>PBS</td>
<td>10/10</td>
</tr>
</tbody>
</table>

Antibody production in immunized mice

As earlier experiments by other workers had shown that antibody to NS1 could be protective in animal models the kinetics of the humoral immune response were studied in our mouse model. NS1-specific IgG antibodies began to appear by day 7 after immunization and rose to a peak by day 10 (Fig. 1). Although these antibody levels were quite high they began to fall soon after to reach levels just above background by day 21. The appearance of IgM followed a similar pattern, but at very low levels, peaking a little earlier than IgG, as expected. IgM could not be detected after day 14, although the levels of IgG and IgM could not be compared directly as the relative sensitivity of the commercial reagents used to measure them could not be determined.

Enhanced protection with conventional vaccine

As conventional inactivated vaccines are expensive and difficult to produce it is of interest to determine whether the protective efficacy of existing stocks of conventional vaccines can be enhanced by supplementing them with Rad viruses. A single batch of TBE vaccine, with a PD50 of 1/114, was examined. When aliquots of this vaccine batch were supplemented with 107 p.f.u. of Rad51, a substantial enhancement in protective efficacy was observed, with a PD50 of 1/1020 being recorded for the mixture of vaccine and recombinant virus.

Table 2. Adoptive transfer experiments in BALB/c mice with immunological material from BALB/c mice immunized with Rad51

Results are from animals receiving material from mice immunized once with Rad51 or PBS and challenged with Absettarov virus as described in Methods.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Survivors after pretreatment with cyclophosphan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisera</td>
<td>4/10† (0/10‡)</td>
</tr>
<tr>
<td>Macrophages + antisera</td>
<td>3/10 (0/10)</td>
</tr>
<tr>
<td>B-cells</td>
<td>4/10 (0/10)</td>
</tr>
<tr>
<td>T-cells</td>
<td>0/10 (0/10)</td>
</tr>
</tbody>
</table>

§ Data from animals receiving material from unimmunized mice.

† Data from animals receiving material from mice immunized with Rad51 in PBS.

Passive protection by adoptive transfer of antibodies and lymphocytes

Other workers have shown that polyclonal and monoclonal antibodies to the NS1 protein of mosquito-borne flaviviruses can protect against virus challenge in experimental murine and primate models. Experiments reported here (Table 2) show that antibodies and B cells from animals immunized with Rads containing the NS1 gene from a tick-borne flavivirus also elicit significant levels of protection. These levels of protection could not, however, be increased by adding macrophages to the preparation even though high levels of TNF were shown to be generated in other experiments (see below) and genetically homogeneous mice were studied.

Measurement of cytokines in vaccinated mice

As NS1 is a non-structural protein it is assumed that its main mode of action in eliciting a protective immune response is through the stimulation of Th1-like cells which in turn...
stimulate B cells involved in the complement fixation pathway and also stimulate CD8+ T cells to produce cytotoxic T cells. Thus, levels of key cytokines in this branch of the immune system were measured in vaccinated mice. Levels of TNF-β and γ-interferon were raised to high levels in these animals (Fig. 2a, b). TNF levels reached a maximum by day 4 and remained high until after day 8, whereas levels of IFN peaked earlier (day 3), but had fallen back to background levels by day 5. Conventional biological assays for total interferon levels (Fig. 2c) gave similar results. Both these cytokines have been shown by other workers to stimulate the complement-fixing pathway, CTL maturation and macrophage activation, and therefore our finding of elevated levels during a protective immune response stimulated by NS1 is consistent with their known roles. IL-2 is associated with the activation of naive CD4+ cells, stimulates Th1-like cells themselves and is in turn involved in their stimulation of Type 1 effector actions. Levels of this cytokine were elevated by day 3 following vaccination, had peaked by day 4 and then rapidly dropped to background levels (Fig. 2d).

Levels of IL-1β, which stimulates macrophage activity and the inflammatory response in general, were also measured. By day 3 following immunization, raised levels of this cytokine were detected (Fig. 2e) and these remained constant for about 4 days, but then returned to background levels.

Immunization with a control adenovirus (Rad35) caused very little elevation in the levels of any cytokines at any time.

Discussion

It is well-established that flavivirus infections elicit an antibody response to both the major virion envelope protein, E, and a major non-structural glycoprotein, NS1 (Hambleton et al., 1983; Gould et al., 1986). Curiously, immune responses to both structural and non-structural proteins appear to play an important role in controlling flavivirus diseases. Purified NS1 protein can protect against yellow fever virus-mediated encephalitis in a mouse model (Schlesinger et al., 1986) and passive transfer of NS1-specific monoclonal antibodies can also
be protective (Schlesinger et al., 1985; Gould et al., 1986). Furthermore, previous studies with a recombinant adenovirus (Rad51) containing the NS1 gene demonstrated strong protection against infection with the parent strain of TBEV (Neudorf) (Jacobs et al., 1992, 1994).

Experiments reported here show that mice immunized with Rad51 are protected not only against the parent strain of TBEV and can enhance the protective efficacy of conventional vaccines, but are also immune to other Western and Far-eastern subtype virus isolates. Similar experiments using conventional commercial vaccines also showed good cross-protection against TBEV isolates from Russia, Western Europe and Eastern Europe (Klockmann et al., 1991; Holzmann et al., 1992). The protection against infection with the ‘R’ virus isolate is especially noteworthy as the ratio between i.p. infectivity and intracranial infectivity is 100 times higher for this isolate than standard TBEV isolates such as Sophyn. In addition, Rad51 gave good levels of protection against infection with Omsk haemorrhagic fever virus, although only low levels of protection were observed after challenge with Negishi virus. The poor protection against Negishi virus infection appears to conflict with our earlier observations (Stephenson et al., 1984) and those of several other workers which show good antigenic homogeneity between TBEVs and louping ill viruses. The E genes of Negishi virus and louping ill virus have been shown by Venugopal et al. (1992) to be very closely related and therefore higher levels of cross-protection would have been predicted. However, the isolate of Negishi virus used in these studies is of low-passage number and comes from the Far-eastern Region of Russia. It is therefore a different isolate from that available in most other laboratories and in our hands has a higher virulence in experimental animals (J. R. Stephenson & A. V. Timofeev, unpublished results).

The high levels of cross-protection observed here are consistent with the sequence and structural conservation previously reported for the NS1 protein of some of the viruses from the TBEV complex (Jacobs et al., 1993), although NS1 gene sequences of several of the TBEV strains used in this study are not currently available.

Adoptive transfer experiments demonstrate that antibodies or B cells from mice immunized with a recombinant virus containing only the NS1 gene from TBEV can protect recipient animals against lethal challenge without the participation of other components of the immune system. Although not all animals were protected these results give independent confirmation that antibodies against this non-structural protein can indeed provide protective immunity. Surprisingly however, macrophages from donor mice did not increase the proportion of survivors among recipients when added with antisera, despite the presence of high levels of macrophage-stimulating cytokines such as TNF, IFN and IL-1 (Beutler & Cerami, 1989). Recently however, Hall et al. (1996) failed to protect experimental animals with the adoptive transfer of antisera from mice vaccinated with the NS1 protein from Murray Valleyencephalitis virus. This apparent conflict could probably be explained by their choice of route for the transfer of the donor sera. These authors used the i.p. route in their study and we also failed to show protection in recipients when the i.p. route of inoculation was used (data not shown). Our earlier results showing that complement-deficient animals can also be protected by NS1 protein (Jacobs et al., 1994) seem at variance with these results, however. Recent results from many laboratories indicate that there is a great deal of redundancy in the immune system which appears to act through a complex system of inter-related pathways (reviewed in Abbas et al., 1996). Therefore a possible explanation, consistent with all the current data, is that induction of complement is indeed the dominant mechanism of protection, but when this pathway is inactivated, other mechanisms such as CTL or NK cells come into play. Indeed, the results reported here show that T cells from immunized mice were protective, but only in animals whose pre-existing immune function had been impaired. It could be assumed therefore that these animals contained suppressor elements which prevented the action of cells from the immune animals. The characterization of these T cells is not reported here, but a detailed study of the nature of the T-cell response after NS1 vaccination is under way.

The cytokine profiles observed in vaccinated animals in these studies are indicative of a strong Th1-like immune response. Three cytokines known to stimulate Th1-like T cells, IFN-γ, IL-2 and TNF, were detected at high levels for several days in vaccinated animals. IFN-γ is known to stimulate the maturation of B cells to produce complement-fixing IgG molecules, the activation of macrophages (Coffman et al., 1993) and the maturation of CD8+ T cells to form CTLs (reviewed in Abbas et al., 1996). IL-2 also stimulates the maturation of CD8+ T cells and has an autocrine function in the activation of naive CD4+ T cells. TNF and IL-1 are also known to stimulate macrophages and function as general effectors of the inflammatory response (Kaufmann, 1993). Thus in Rad51-vaccinated mice, the raised levels of cytokines known to activate T cells and B cells are in good correlation with the results of the adoptive transfer experiments also reported here. Furthermore, the single-peak curves for all the cytokines and antibodies studied give no evidence that Rad51 persists in the immune animals. The characterization of these T cells is not reported here, but a detailed study of the nature of the T-cell response after NS1 vaccination is under way.

The production of an inflammatory immune response which efficiently removes infected cells can be counter-productive if allowed to develop unregulated. TNF is known to damage myelin and oligodendroglia (Robbins et al., 1987) and recently it was shown that TNF, together with activated T cells, can cause focal breakdown of the blood–brain barrier (Spies et al., 1995). Thus, if TNF activation is found during the development of TBEV-induced encephalitis it could add to our understanding of TBE pathogenicity.

The demonstration here that Rads containing the NS1 gene can induce a good protective immune response, which is
associated with the stimulation of cytokines associated with a Th1-like response, suggests that vaccines based on this protein should not only be unable to induce ADE, but also should be free of allergic reactions and related side-effects.

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References


Protection against TBE by recombinant NS1


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