Recombinant baculoviruses expressing yellow fever virus E and NS1 proteins elicit protective immunity in mice

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Recently, we showed that yellow fever virus (YFV) E and NS1 proteins in Spodoptera frugiperda cells infected with a recombinant baculovirus are similar, if not identical to those produced during YFV infection. To study the role of E and NS1 in the induction of protective immunity against fatal YFV challenge, these viral antigens were expressed either alone or in tandem via recombinant baculoviruses Ac-E.NS1, Ac-E1 and Ac-NS1. Swiss mice were immunized with lysates of insect cells infected with the recombinant baculoviruses. Solid protection against lethal YFV encephalitis was achieved after immunization with cell lysates containing the E protein with or without the NS1 protein. Mice inoculated with recombinant protein NS1 alone were not significantly protected but showed an increased survival time. Recombinant E protein expressed alone or in tandem with NS1 elicited a low but significant level of neutralizing antibodies. Although protein NS1 synthesized by recombinant baculovirus expressing E plus NS1 was more immunogenic than that expressed alone, neither strategy induced NS1-specific antibodies with complement-mediated cytolytic activity.

The flavivirus family includes viruses that cause human diseases such as yellow fever (YF), dengue fever (DEN), Japanese encephalitis (JE) and tick-borne encephalitis. Virological and molecular studies have been developed recently that could lead to the development of safe and potent flavivirus vaccines (Stephenson, 1988).

Two YFV proteins show great promise as candidates for subunit vaccines: the envelope (E) protein and the non-structural protein (NS1). The E protein is the major target of the protective host immune response; it is present on the surface of the virion and is responsible for virion adsorption to the host cells, haemagglutination and reactivity with neutralizing antibodies (Schlesinger et al., 1983; Buckley & Gould, 1985). The NS1 protein is referred to as the soluble complement-fixing antigen (Brandt et al., 1970). It was shown to be secreted from YFV-infected primate cells (Putnak & Schlesinger, 1990; Desprès et al., 1991).

Mice immunized with immunoaffinity-purified E or NS1 proteins obtained from primate cell lysates infected with YFV strain 17D were shown to be protected against lethal YFV challenge (Schlesinger et al., 1985; Brandriss et al., 1990), indicating that the solubilized major antigens of YFV can protect mice following immunization. Monoclonal antibodies (MAbs) directed against protein E were also shown to protect passively against a lethal challenge with infectious virus (Brandriss et al., 1986; Gould et al., 1986). NS1-specific antibodies alone are also capable of passively protecting animals against a fatal encephalitis caused by challenge with YF or DEN viruses (Gould et al., 1986; Schlesinger et al., 1985, 1986; Henchal et al., 1988). Since protection conferred by NS1-specific MAbs correlated with their ability to fix complement and to induce complement-mediated lysis, Schlesinger et al. (1985) suggested that the protection elicited by the NS1 protein may result from the destruction of infected cells prior to release of the progeny virus. Schlesinger et al. (1985, 1986, 1990) proposed that immune recognition of NS1 and consequent cytolysis afford an alternative to virion neutralization by antibodies in protection against flavivirus infection. These data support the idea that the non-structural NS1 protein could be used as a component of a subunit vaccine against flavivirus infection (Gibson et al., 1988).

To investigate the role of E and NS1 in protection against YFV infection, expression of these two major antigens has been achieved in a recombinant Autographa californica nuclear polyhedrosis baculovirus (AcNPV) system yielding the proteins either alone (Ac-E1 and Ac-NS1) or in tandem (Ac-E.NS1) (Fig. 1). Recombinant proteins E and NS1 expressed by Ac-E.NS1 were
Fig. 1. Sequence organization of YFV polypeptides expressed by recombinant baculoviruses. Gene organization of YFV is presented at the top. Numbers indicate the positions of the N-terminal amino acid residue of the individual E, NS1 and NS2a polypeptides in the YFV polyprotein. Transfer vector pVL 941-poly was used to insert the YFV cDNA into the genome of baculovirus (Després et al., 1991). The recombinant baculovirus Ac-E.NS1 has already been reported (Després et al., 1991). The recombinant baculoviruses Ac-E1 and Ac-NS1 contain the 17D YFV sequences isolated from plasmids pSV-E or pSV-NS respectively (Després et al., 1990). Structural map of translated polypeptides expressed by Ac-E.NS1, Ac-E1 and Ac-NS1 with numbers referring to the amino acids that derive from the YFV polyprotein. Additional amino acid residues to the N and C termini of the translated YFV related polypeptides are indicated. The dark shaded areas indicate DNA encoding YFV N-terminal hydrophobic regions; hatched areas indicate DNA encoding the YFV C-terminal signal region. Downward arrows indicate cleavage sites for signal peptidase.

shown to be similar, if not identical, to those produced by YFV infection (Després et al., 1991). As shown in Fig. 2, proteins E and NS1 were synthesized in insect cells infected with recombinant baculoviruses Ac-E1 or Ac-NS1 respectively. The recombinant protein expressed by Ac-NS1 migrated as a tunicamycin-sensitive closely spaced doublet containing N-linked glycans, possibly processed to the trimannosyl core without further elongation as is the case for the recombinant NS1 protein expressed by Ac-E.NS1 (Després et al., 1991).

Recently, Shiu et al. (1991) described a recombinant baculovirus-expressed 54K E protein which is antigenically indistinguishable from the authentic 17D YF vaccine viral protein. The antigenicity of our recombinant protein E expressed alone was assessed using a panel of MAbs directed against the YFV E protein. Neutralizing E-specific MAbs 2C9, 2D12, 2E10, 4E8 and 5E3 (Schlesinger et al., 1983) as well as MAbs 612, 825, 843 and 864 (Gould et al., 1985; Buckley & Gould, 1985) immunoprecipitated the E protein expressed by recombinant baculovirus Ac-E1 which lacks the carboxyterminal transmembrane anchor domain. MAbs 825 and 864 were shown to confer passive protection in mice lethally challenged with live 17D YFV (Gould et al., 1986). Recombinant NS1 expressed by Ac-NS1 possessed all the epitopes recognized by the NS1-specific MAbs 1A5, 2D10, 4E3, 2G2 and 8G4 (Schlesinger et al., 1983). MAbs 1A5 and 8G4 were shown to exhibit a high titre of complement-fixing activity, to lyse YFV-infected mammalian cells via complement and to protect mice passively against YFV fatal encephalitis (Schlesinger et al., 1985). Moreover, MAb 1A5 was shown to reduce the level of viral spread in tissue culture (Schlesinger et al., 1990). All of the NS1-specific MAbs tested were capable of reacting with the unglycosylated form of NS1 (tunicamycin-treated form of NS1) indicating that N-glycans are not required for the expression of the major epitopes. These results demonstrated that the majority of immunodominant epitopes associated with YFV E and NS1 proteins were also present in the YFV-related antigens expressed in infected Spodoptera frugiperda (Sf) cells.

Coomassie blue staining of the gels showed that the amounts of recombinant E or NS1 alone, but not E plus NS1, expressed in Sf cells reached an almost similar level of 5 µg per million infected cells at 30 h post-infection. Insect cells infected with Ac-E.NS1 did not produce enough recombinant proteins E and NS1 to be visualized by Coomassie blue staining. Oligomerization of YFV or recombinant NS1 was shown to give rise to a heat-labile homoglycoprotein with an Mr of 72K (Després et al.,

Fig. 2. RIA of baculovirus-expressed YFV proteins. [35S]Methionine-labelled Vero cell lysates infected with 17D YFV (17D, lanes 1 and 2) or the Sf9 cell lysates infected with wild-type baculovirus (AcNPV, lane 5), Ac-E. NS1 (lane 6), Ac-E1 (lane 3), Ac-NS1 (lanes 7 and 8) or mock-infected (Sf9, lane 4) were immunoprecipitated with a mouse immune serum directed against 17D YFV as previously described (Després et al., 1991). Tunicamycin (5 µg/ml) was present (lanes 2 and 8) or absent (lanes 1 and 7) throughout the labelling period. YFV proteins are indicated at the left. The positions of the protein markers are indicated. Proteins were analysed in a 15% SDS–polyacrylamide gel.
YFV-infected primate cells. Although the dose of cells primate cell lysates contributes to protection. A fourth, positive control group of mice was immunized with live negative control group, mice were immunized with (0.5 × 10^6) lysates from insect cells infected with wild-type AcNPV. The possibility that residual live virus from the infected 2 week interval, without adjuvant, by the intraperitoneal injecting 3-week-old Swiss mice with Sf cell lysates infected with Ac-NS1, Ac-E1 or Ac-NS1. As a control group, mice were immunized with lysates from insect cells infected with wild-type AcNPV. A third group of mice was immunized with lysates of 17D YFV-infected primate cells. Although the dose of cells (0.5 × 10^6) used for immunization contained less than 10^3 p.f.u. of infectious virus, we cannot rule out the possibility that residual live virus from the infected primate cell lysates contributes to protection. A fourth, positive control group of mice was immunized with live 17D YFV. All the animals were immunized twice with a 2 week interval, without adjuvant, by the intraperitoneal (i.p.) route. One week after the last immunization, mice were challenged intracerebrally with 100 LD₅₀ of live 17D-204 Pasteur vaccine strain grown in SW13 cells. Mice were observed for 25 days for signs of encephalitis and the mortality rate was recorded daily. The protection rate in each group of immunized mice was computed by standard chi-square analysis.

Following intracerebral (i.c.) inoculation of live 17D virus, all mice (20/20) immunized with Vero cell lysates infected with 17D YFV were protected and 16 of 19 animals vaccinated with live 17D YFV survived. As expected, immunization with AcNPV was not protective since 18 of 19 Swiss mice immunized with lysates from insect cells infected with AcNPV showed symptoms of central nervous system (CNS) disease which progressed to death after an average period (mean ± s.d.) of 12.6 ± 0.9 days (Fig. 3).

Fig. 3. Mouse protection assay. The SF9 cells were infected with wild-type baculovirus AcNPV (●) or recombinant baculoviruses (○, Ac-E-NS1; □, Ac-E1; ▲, Ac-NS1) at a multiplicity of 10. The infected insect cells were harvested at 30 h p.i., washed twice in cold PBS and frozen at −20°C. To obtain the cell lysates used for immunization, frozen cells were thawed rapidly at 37°C for 5 min. Swiss mice immunized with a double i.p. inoculation with lysates from infected SF9 cells were challenged i.c. with a dose of 100 LD₅₀ live 17D YFV. The proportion of mice protected was evaluated at each day post-challenge. In preliminary experiments, we have shown that Swiss mice immunized with lysates from 0.5 × 10^6 insect cells infected with recombinant baculovirus Ac-E. NS1 were partially protected (50%) against lethal i.c. challenge. Following immunization with lysates from 2.5 × 10^6 insect cells infected with Ac-E. NS1, all of 25 YFV-challenged Swiss mice survived (Fig. 3) although one of them showed symptoms of CNS disease which progressed to permanent hind leg paralysis. These results suggest that the degree of protection afforded by immunization with E plus NS1 was directly related to the amount of the YFV antigens inoculated into the mice (P < 0.001). As shown in Fig. 3, immunization of mice with lysates from 0.5 × 10^6 insect cells infected with Ac-E1 induced solid protection against fatal YFV encephalitis (17/19) (P < 0.001). The protection rate of mice inoculated with recombinant protein E alone was not significantly different from that of the mice immunized with recombinant baculovirus expressing E plus NS1 (P > 0.3). These findings confirm that protein E is the major protective antigen of YFV. In contrast to the 90% survival rate observed with protein E alone, only 20% (5/25) of YFV-challenged Swiss mice survived following immunization with lysates from 0.5 × 10^6 insect cells infected with Ac-NS1 (Fig. 3). The low protection rate of mice immunized with recombinant NS1 alone was not significantly different from that of the negative control group (P > 0.3). Although recombinant NS1 alone failed to induce significant protection, it elicited an immune response which appeared to delay YFV spread in CNS tissues as revealed by the increased survival time (average day of death of 16 ± 1) when compared with AcNPV (Fig. 3). Moreover, surviving mice immunized with recombinant NS1 alone did not develop symptoms of CNS disease following YFV challenge.

These results contrast with the significant protection against 17D YFV challenge observed in BALB/c mice immunized with recombinant vaccinia virus expressing 17D YFV NS1 to NS2B (Putnak & Schlesinger, 1990). Passive protection against YFV challenge by NS1-specific MAbs was also observed and was directly related to virus neurovirulence (Gould et al., 1986). Furthermore, immunization with Escherichia coli-derived recombinant NS1 was shown to protect mice against one of the least virulent strains of 17D YFV (Cane & Gould, 1988). Although we cannot exclude the possibility that the protective capacity of NS1 is related to the expression vector system or to the choice of mouse strain used for immunization, it could be that the different degree of protection observed by these groups depends on the degree of neurovirulence of the 17D YFV strain used for challenge. It is also possible that polypeptide NS2A contributes to the protective capacity of NS1.
This was suggested by Falgout et al. (1990) who showed that maximal protective efficacy against DEN 4 virus challenge required immunization with the DEN 4 NS1 protein synthesized by a recombinant vaccinia virus expressing NS1 plus NS2A. Further experiments must be designed to determine whether NS2A contributes to stimulation of the protective efficacy of NS1.

To analyse their serological response, inoculated mice were bled 2 days prior to challenge and those which survived were bled again 6 weeks after challenge. Sera obtained from each group of mice were pooled and those which survived were bled again 6 weeks after challenge. Sera from mice immunized with 17D YFV. Although recombinant E induced the production of neutralizing antibodies, it was less immunogenic than the authentic E protein as measured by the RIP assay (data not shown). Similar observations had been reported with recombinant baculoviruses expressing the DEN 4 virus E protein (Zhang et al., 1988; Lai et al., 1989, 1990). It is possible that the weak immunogenicity is due to the fact that the recombinant E protein does not oligomerize as does the E protein present in mature virions (Heinz & Kunz, 1980). By the RIP assay, we have repeatedly observed that sera of mice immunized with Ac-E. NS1 reacted with the YFV NS1 protein whereas protein NS1 from cell lysates infected with Ac-NS1 failed to induce specific antibodies at a significant level. This finding suggests that NS1 protein produced by the recombinant baculovirus expressing E plus NS1 was substantially more immunogenic than the same protein expressed alone. The failure of NS1 alone to elicit protective immunity might be related to this difference in immunogenic potential.

Dimers of DEN 2 virus NS1 protein have been shown to be more antigenic and immunogenic than their monomeric counterpart (Falconar & Young, 1990). The immunogenic difference of NS1 was not correlated with the presence of NS1 oligomers since the gp72 form was observed in both cases. A possible explanation would be that the gp100 precursor to E and NS1 which was detected in insect cells infected with Ac-E.NS1 (Després et al., 1989, 1990). It is possible that the weak immunogenicity of NS1 was not correlated with the presence of NS1 oligomers since the gp72 form was observed in both cases. A possible explanation would be that the gp100 precursor to E and NS1 which was detected in insect cells infected with Ac-E. NS1 (Després et al., 1991), has a strong immunogenicity by itself. Our preliminary results confirm that the uncleaved gp100 precursor expressed by a recombinant baculovirus induced NS1-specific antibodies. Moreover, it has been proposed that antibodies to NS1 may serve to eliminate flavivirus-infected cells by the mechanism of complement-mediated lysis. As shown in Table 1, however, sera from mice immunized with insect cell lysates fermented with Ac-E.NS1 or Ac-NS1 did not exhibit significant CMC activity when compared with those from mice immunized with 17D YFV. From this result, we were unable to correlate protection with the presence of NS1-specific antibodies exhibiting CMC activity. Our results might be interpreted as being consistent with those showing that DEN 2 virus NS1-specific MAbs without CMC activity were capable of protecting DEN 2 virus-challenged mice (Henchal et al., 1988). However we propose that other mechanisms may be involved in immune recognition of NS1 on the surface of flavivirus-infected cells. We suggest that immune recognition of recombinant protein NS1 might delay YFV spread through a mechanism of cell killing such as antibody-dependent cytotoxicity. Thus, the presence of NS1 at the cell surface might attract cytotoxic cells limiting the virus spread by lysis of YFV-infected cells.

### Table 1. Immune response to E and NS1 expressed by recombinant baculoviruses

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Percentage surviving</th>
<th>PRNT&lt;sub&gt;50&lt;/sub&gt; Pre-challenge</th>
<th>PRNT&lt;sub&gt;50&lt;/sub&gt; Post-challenge</th>
<th>CMC (%) Pre-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live 17D (Control)</td>
<td>85</td>
<td>1:60</td>
<td>1:400</td>
<td>34</td>
</tr>
<tr>
<td>Vero/17D (Control)</td>
<td>100</td>
<td>1:10</td>
<td>1:600</td>
<td>60</td>
</tr>
<tr>
<td>AcNPV (Control)</td>
<td>5</td>
<td>&lt;1:10</td>
<td>NA†</td>
<td>6</td>
</tr>
<tr>
<td>Ac-E. NS1</td>
<td>100</td>
<td>1:10</td>
<td>1:1600</td>
<td>5</td>
</tr>
<tr>
<td>Ac-E1</td>
<td>90</td>
<td>1:20</td>
<td>1:3200</td>
<td>1:5</td>
</tr>
<tr>
<td>Ac-NS1</td>
<td>20</td>
<td>&lt;1:10</td>
<td>1:800</td>
<td>8</td>
</tr>
</tbody>
</table>

* Antibodies from mouse immune sera obtained before (pre-challenge) or after YFV challenge (post-challenge) were tested for serological data. The serological response of each group of mice immunized with 17D YFV (live 17D) or with Vero cell lysates infected with 17D YFV (Vero/17D) or with Sf9 cell lysates infected with wild-type baculovirus (AcNPV) or with recombinant baculoviruses that survived the challenge is shown. The neutralizing antibody titre was expressed as the dilution of the serum that yielded a 50% reduction in the infectivity of 100 p.f.u. 17D YFV as determined by the PRNT<sub>50</sub>. A complement-dependent, antibody-mediated cytotoxicity assay was performed with immune serum employing the procedure described by Schlesinger et al. (1985). CMC activity in the sera diluted 10-fold obtained before challenge was measured by specific <sup>51</sup>Cr release (percentage lysis) from labelled 17D YFV-infected SW13 cells.

† NA, Not applicable.

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This was suggested by Falgout et al. (1990) who showed that maximal protective efficacy against DEN 4 virus challenge required immunization with the DEN 4 NS1 protein synthesized by a recombinant vaccinia virus expressing NS1 plus NS2A. Further experiments must be designed to determine whether NS2A contributes to stimulation of the protective efficacy of NS1.

To analyse their serological response, inoculated mice were bled 2 days prior to challenge and those which survived were bled again 6 weeks after challenge. Sera obtained from each group of mice were pooled and assessed for neutralization activity, complement-mediated cytolysis (CMC) activity and complement precipitates (RIP) assay. The plaque reduction neutralization test (PRNT<sub>50</sub>) indicated that the neutralizing antibody level in pre-challenge sera of mice immunized with 17D YFV was lower than that obtained with live 17D vaccine (1:10 compared to 1:60) (Table 1). Thus, it appeared that the immunogenic capacity of the intracellular protein E to stimulate the production of neutralizing antibodies was lower than that of live 17D. When compared with the intracellular YFV E protein, recombinant E protein expressed by Ac-E. NS1 or Ac-E1 elicited similar levels of virus neutralizing antibodies (Table 1). Together, these results indicate that a low level of neutralizing antibodies seems to be sufficient to protect mice against an i.c. inoculation with a fatal dose of YFV. Although recombinant E induced the production of neutralizing antibodies, it was less immunogenic than the authentic E protein as measured by the RIP assay (data not shown).
In the post-challenge sera of mice immunized with recombinant baculoviruses expressing protein E, we observed an enhanced level of E-specific antibodies, as measured by the RIP assay and PRNT50 (Table 1). The titre of neutralizing antibodies was even higher than that obtained with the authentic E protein. This finding confirms that primed lymphoid cells expressing E-specific antibodies were present in mice immunized with recombinant protein E and were stimulated following challenge with live 17D YFV. The presence of NS1-specific antibodies in the post-challenge sera of mice immunized with recombinant baculovirus expressing E alone (data not shown) indicated that viral replication occurred following i.c. inoculation with live 17D YFV.

In conclusion, solid protection against YFV challenge required immunization of mice with cell lysates containing protein E and was correlated with the induction of YFV neutralizing antibodies albeit at a low level. Immunization with cell lysates containing NS1 alone did not confer significant protection against fatal YFV encephalitis but protein NS1 was shown to delay YFV spread, suggesting that it could play a role in protection against virus infection. Our data support the idea that immune protection against flavivirus infection may be optimized by the synergistic interaction of the E and NS1 antigens. Thus, our recombinant baculoviruses expressing E in tandem with NS1 could be used as a source of antigens for the development of a subunit vaccine against YFV. To investigate further the role of the E and NS1 proteins in induction of the protective immune response, purification of these proteins is required along with studies of their respective protective capacity.

Note added in proof. A recombinant baculovirus expressing the uncleaved precursor gp100 was constructed in which the VCA tripeptide cleavage site upstream of NS1 has been changed into FYV by directed mutagenesis. Similar to the results obtained with Ac-E.NS1, immunization with infected cell lysates containing the mutant gp100 induced complete protection against YFV encephalitis in mice and production of neutralizing antibodies (1/10) as well as NS1-specific antibodies.

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References


Short communication


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