Reduction of Yellow Fever Virus Mouse Neurovirulence by Immunization with a Bacterially Synthesized Non-structural Protein (NS1) Fragment

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SUMMARY

Part of a yellow fever virus-specified non-structural protein (NS1) was expressed in Escherichia coli as a fusion protein with β-galactosidase. Immunization of mice with this partially purified NS1-β-galactosidase fusion protein induced yellow fever virus-specific antibodies and provided some protection against intracerebral challenge with the virus.

INTRODUCTION

Yellow fever (YF) virus is the type species of the family Flaviviridae (Westaway et al., 1985) which contains many other human pathogens including Japanese encephalitis virus and the four dengue virus (DEN) serotypes. Mouse protection experiments using passively administered monoclonal antibodies showed that immunity to a non-structural glycoprotein (NS1) produced in YF virus-infected cells can protect mice against challenge with some strains of YF virus (Schlesinger et al., 1985; Gould et al., 1986). Moreover, active immunization with purified NS1 can protect both mice and rhesus monkeys in the absence of virus-neutralizing antibodies (Schlesinger et al., 1985, 1986). However, the purified NS1 protein was derived from virus-infected cells, and thus there is a slight possibility that trace amounts of contaminating virion envelope glycoprotein in the NS1 protein preparations contributed to the observed protection (Schlesinger et al., 1986).

This report describes the synthesis in Escherichia coli of a fragment of YF virus NS1 protein in the form of a fusion protein with β-galactosidase, and the use of this fusion protein to immunize mice against YF virus. NS1 protein thus produced in bacteria is certain to be free of other virus-coded components, and can be readily produced in large quantities.

METHODS

Preparation of plasmid DNA. Small scale plasmid screenings were carried out by the method of Holmes & Quigley (1981). Plasmid DNA for cloning was prepared by caesium chloride density gradient centrifugation of cleared lysates (Guerry et al., 1973).

Cloning. Restriction endonucleases (Bethesda Research Laboratories) and DNA ligase (Boehringer) were used as specified by the manufacturers. A cDNA clone derived from YF virus strain 17D was kindly provided by Dr C. M. Rice (Washington University, Saint Louis, Miss., U.S.A.). This clone carried YF virus nucleotide sequences from 154 to 3718 which encode most of the virus structural proteins together with the NS1 protein (Rice et al., 1985). A HindIII fragment (nucleotides 2574 to 2844) from the NS1 region of the cDNA clone was subcloned into expression plasmid pUR292 using standard techniques (Maniatis et al., 1982). The pUR series of vectors has been constructed to allow insertion of cDNA in all reading frames at the 3' end of the lacZ gene, while retaining β-galactosidase activity (Ruther & Muller-Hill, 1983). The appropriate pUR vector was chosen by reference to the nucleotide sequence of YF virus (Rice et al., 1985). The recombinant plasmids were used to transform E. coli BMH17-18 by standard procedures (Cohen et al., 1972), and transformants were grown on L agar containing 100 μg/ml ampicillin.

Protein analyses. Cultures were grown to mid-log phase and then induced with 1 mM-isopropyl β-D-thiogalactoside (IPTG) for 1 to 3 h. Total bacterial lysates were prepared by suspending pelleted cells in SDS...
Purification of fusion protein. Cultures were grown to mid-log phase and then induced overnight with 1 mM-IPTG. Both NS1-β-galactosidase and normal β-galactosidase were partially purified from cells lysed by lysozyme and non-ionic detergent by the method of Watson et al. (1985). All preparations were then examined by SDS-PAGE analysis.

Immunoblotting. Bacterial lysates or purified fusion proteins were subjected to SDS–PAGE analysis and then electrophoretically transferred to nitrocellulose membranes. The blots were incubated with blocking buffer (5% fat-free milk powder, 0.2% Tween 20 in phosphate-buffered saline; PBS) for 1 h at room temperature, then with anti-YF virus antibody (see below) diluted 1:100 in PBS with 1% bovine serum albumin and 0.2% Tween 20 and pre-absorbed with E. coli lysate, overnight at 4 °C. Following washes in PBS with 0.2% Tween 20, the blots were incubated with affinity-purified horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Bio-Rad) for 2 h, washed, and developed with diaminobenzidine (Sigma) with hydrogen peroxide in PBS.

Preparation of immune antiserum to YF17D. A plaque-purified avirulent variant of YF17D was inoculated intracerebrally (0.01 ml) into 3- to 4-week-old randomly bred female mice (strain TO; Tuck & Sons, Battlesbridge, U.K.), and pooled antisera were collected from survivors after 21 days. By indirect immunofluorescence tests this antiserum reacted with YF17D-infected Vero cells at dilutions of up to 1:5000.

Preparation of hyperimmune sera to NS1-β-galactosidase. Three- to 4-week-old TO mice were inoculated intramuscularly with 50 μg fusion protein in an equal volume of Freund’s complete adjuvant. Five consecutive intradermal inoculations of 50 μg fusion protein without adjuvant were given at weekly intervals.

Mouse protection experiments. Groups of 3- to 4-week-old female TO mice were inoculated intramuscularly with 50 μg of NS1-β-galactosidase or β-galactosidase in Freund’s complete adjuvant, followed by intradermal inoculations of 50 μg of appropriate protein without adjuvant 1 week later. After a further week, the mice were challenged intracerebrally with an estimated 100 LD₅₀ (as estimated in newborn mice) per mouse of appropriate virus. Virus strains used were the French neurotropic vaccine (FNV), YF17D from PHLS Porton Down, Salisbury, U.K., (P17D), and a medium-sized plaque virus derived from Wellcome Laboratories Arilvax YF17D (RMP) (Liprandi, 1981).

Antibody detection. Anti-NS1 antibodies were titrated by immunofluorescence and complement fixation tests (CFT) using standard techniques.

RESULTS

Synthesis of NS1-β-galactosidase fusion protein

Clones that contained the required plasmid fragments were analysed by SDS–PAGE, before and after induction with IPTG. Of these, about half were found to express a fusion protein of about 125K following addition of IPTG. The other clones were assumed to have the inserted DNA in the wrong orientation for correct expression. Fusion proteins were produced in great abundance (Fig. 1), and accumulated as insoluble aggregates which were readily enriched by centrifugation through a sucrose cushion following lysis by lysozyme and non-ionic detergent. SDS–PAGE analysis of these preparations showed them to be almost completely free of other bacterial proteins (Fig. 2). The fusion protein usually occurred as a double band, as has been previously reported for this vector (Matlashewski et al., 1986), the lower band probably representing a cleavage product. Normal β-galactosidase also accumulated as insoluble aggregates presumably as a result of the large quantities being produced. Following induction with IPTG, clones expressing fusion proteins also showed novel protein bands sized 29K and 32K, while clones expressing normal β-galactosidase only produced the 29K band. Both of these novel protein bands appeared in the partially purified insoluble fusion protein preparations and possibly represent cleavage products of these proteins.

One clone which expressed a fusion protein was further examined by immunoblotting; the probe antibody was a mouse antisera derived using YF17D. Following extensive absorption with E. coli extracts and additional partially purified β-galactosidase, this antisera reacted with the fusion protein but only faintly with β-galactosidase from extracts of cells carrying the pUR292 plasmid without an insert (Fig. 2). However, no reaction could be unequivocally demonstrated between a number of NS1-specific monoclonal antibodies (Gould et al., 1986) and the fusion protein. No reaction was observed between the polyclonal antisera and the 29K and 32K polypeptides mentioned above. Taking into account the defined restriction fragment inserted into the expression vector together with the reaction in immunoblotting with a
Immunization of mice with YF virus NS1

Fig. 1. SDS–PAGE analysis of fusion protein production by E. coli following addition of IPTG for 0 h (lanes 1, 5), 1 h (lanes 2, 6), 2 h (lanes 3, 7) and 3 h (lanes 4, 8). Lanes 1 to 4 show NS1–β-galactosidase production (arrowed); lanes 5 to 8 are controls showing normal β-galactosidase production. Coomassie Brilliant Blue staining. Molecular weight (×10⁻³) markers are shown on the left.

polyclonal YF virus-specific antiserum, it was concluded that the fusion protein contained a YF virus-coded polypeptide fragment of NS1 protein.

**Immunization of mice with NS1–β-galactosidase**

Mice hyperimmunized with partially purified NS1–β-galactosidase developed antibodies that showed virus-specific fluorescence in YF virus-infected Vero cell cultures. The titre of this antibody was estimated at 1:512 by serial dilution tests. Fluorescence was mainly confined to the perinuclear area of the infected cells, with some diffuse staining of the cytoplasm (Fig. 3). There was a marked prozone effect with all the antisera tested, the sera appearing to produce no reaction up to a dilution of about 1:32. In CFT the hyperimmune sera showed titres of between 1:64 and 1:256 when using YF17D-infected suckling mouse brain antigen. These sera also reacted in CFT against non-infected suckling mouse brain antigen but gave four- to eightfold lower titres.

**Protection of mice against lethal challenge with YF virus**

The results of the protection experiments are summarized in Table 1. The proportion of mice dying following challenge with the RMP strain of YF virus was significantly reduced in mice which had received two inoculations of NS1–β-galactosidase fusion protein, when compared
Fig. 2. SDS-PAGE and immunoblotting of partially purified NS1-β-galactosidase and normal β-galactosidase. Partially purified NS1-β-galactosidase (lanes 1, 3) and normal β-galactosidase (lanes 2, 4) were subjected to SDS-PAGE. Lanes 1 and 2 were stained with Coomassie Brilliant Blue; lanes 3 and 4 were immunoblotted using a YF virus-specific polyclonal antiserum. Molecular weight markers (×10^-3) are shown on the left.

Table 1. Effect of immunization with NS1-β-galactosidase on intracerebral challenge with various strains of YF virus

<table>
<thead>
<tr>
<th>Virus</th>
<th>Immunogen</th>
<th>No. mice dead/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>YF17D RMP</td>
<td>β-galactosidase</td>
<td>18/50</td>
</tr>
<tr>
<td>YF17D RMP</td>
<td>NS1-β-galactosidase</td>
<td>8/50</td>
</tr>
<tr>
<td>YF FNV</td>
<td>β-galactosidase</td>
<td>10/10</td>
</tr>
<tr>
<td>YF FNV</td>
<td>NS1-β-galactosidase</td>
<td>10/10</td>
</tr>
<tr>
<td>YF P17D</td>
<td>β-galactosidase</td>
<td>10/10</td>
</tr>
<tr>
<td>YF P17D</td>
<td>NS1-β-galactosidase</td>
<td>10/10</td>
</tr>
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with mice that had been immunized with control protein (P < 0.02). However, immunization with NS1-β-galactosidase conferred no protection against intracerebral challenge with the more virulent FNV and P17D strains of YF virus. These results are consistent with the previous observations of Gould et al. (1986) that antibody against NS1 protects mice only against infection with the least virulent strains of YF virus.
DISCUSSION

This paper describes the synthesis by E. coli of a fusion protein comprising a fragment of YF virus NS1 linked to β-galactosidase. This fusion protein was produced in great abundance and reacted in immunoblot analyses with YF virus-specific antibody. It also induced a YF virus-specific antibody response after injection into mice. Furthermore, immunization of mice with this fusion protein provided some protection against challenge with YF virus (strain RMP). Lack of complete protection probably reflects the very short time scale of the immunization programme, early challenge following immunization being necessary because mice become resistant to YF virus after 5 to 6 weeks of age (unpublished observations). Failure of complete protection against YF virus infection is possibly also due to the fusion protein containing approximately only one-quarter of the total NS1 protein sequence which may not represent that part of the protein most significant in inducing protective immunity. The level of protection described is very similar to that shown against DEN-2 following immunization with NS1 purified from DEN-2-infected cells (Schlesinger et al., 1987). Lack of protection against other strains of YF virus may be due to their greater virulence and shorter incubation periods. On the other hand different strains may express an antigenically different NS1 protein or it may be expressed in a different manner at the surface of infected cells. A similar situation was described by Schlesinger et al. (1987) who found that mice immunized with DEN-2 NS1 were protected against DEN-2 but not against DEN-1, even though there was cross-reactivity in a CFT between the DEN-2 NS1 antiserum and the other dengue strains.

The mechanism of the protection afforded by NS1 antibodies to flaviviruses is not understood. NS1 protein has been detected on the surface of infected cells (Gould et al., 1986; Westaway & Goodman, 1987) and the ability of anti-NS1 monoclonal antibodies to confer protection correlates with their ability to fix complement and to induce complement-mediated lysis (Schlesinger et al., 1985). Protection may therefore be provided by complement-mediated lysis of virus-infected cells. In animals that have been immunized with NS1, there may also be a contribution of T cell-mediated immunity to protection. With the availability of large quantities
of virus-coded components readily produced by bacteria, it should be possible to explore, in greater depth, the mechanism of the protection induced by immunization with NS1 protein. Understanding the mechanisms involved in immunity to flaviviruses should contribute to a rational design of a synthetic vaccine against flaviviruses, particularly those against which it has not yet been possible to develop a successful vaccine.

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REFERENCES


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