Passive protection studies in mice with monoclonal antibodies directed against the non-structural protein NS3 of dengue 1 virus

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Antibody-mediated enhancement of dengue virus replication is thought to be a mechanism contributing to the pathogenesis of dengue haemorrhagic fever and dengue shock syndrome. Enhancement is associated with antibodies to structural components of the virus. To circumvent the problem of immune enhancement, studies to identify protective antigens of dengue virus have involved non-structural proteins. Passive and active protection against lethal dengue virus infection in mice have been demonstrated with the non-structural protein NS1. In this study, the dengue virus non-structural protein NS3 was examined in passive protection studies with monoclonal antibodies prepared against NS3 of dengue 1 virus (Hawaiian). Five monoclonal antibodies that were authenticated to be reactive to NS3 were used to immunize 13- to 14-day old mice intraperitoneally. Thereafter, the mice were challenged intracerebrally with 100 LD₉₀ of neurotropic dengue 1 virus and the survival indices of the mice were calculated. Significant decreases in survival indices (P<0.05), indicating increases in survival times were observed with four of five monoclonal antibodies tested. Monoclonal antibodies to NS3 of dengue 1 virus are able to increase the survival time of mice challenged with a lethal dose of dengue 1 virus, although the mechanism remains to be defined.

The four serotypes of dengue viruses are positive-strand RNA viruses belonging to the flavivirus family. Infection with these viruses may result in a mild febrile illness or the more severe dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The dengue virus genome consists of a single open reading frame of 10.17 kb (Deubel et al., 1988) which is translated into a precursor polyprotein (Rice et al., 1986). Proteolytic cleavages of the polyprotein result in formation of the core, membrane and envelope proteins and the non-structural proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5.

At present, there are no dengue vaccines available although several live attenuated dengue 1, 2 and 4 candidate vaccines are undergoing clinical trials (Bhamaрапravati et al., 1987; Bhamaрапravati, 1989). A major concern in dengue vaccine development is immune enhancement which is associated with antibodies to structural components of the virus and believed to be a mechanism contributing to the pathogenesis of DHF/DSS (Halstead, 1988). Ideally, a dengue vaccine should protect against all four dengue serotypes. Non-structural proteins as candidate vaccines not only circumvent the problem of immune enhancement but also are well conserved amongst the flaviviruses (Rice et al., 1985; Deubel et al., 1988) and therefore have the potential of conferring immunity against all four serotypes of dengue.

Passive transfer of monoclonal antibodies to NS1 of yellow fever virus has been shown to confer immunity in mice (Schlesinger et al., 1985; Gould et al., 1986). In passive protection studies, mixtures of monoclonal antibodies to the non-structural protein NS1 were shown to protect mice from lethal challenge with dengue virus (Henchal et al., 1988; Falconar & Young, 1989). Schlesinger et al. (1987) demonstrated protection in mice by immunization with NS1 protein, but protection in monkeys could not be demonstrated (Zhang et al., 1989). This appears to argue for a multi-component subunit vaccine comprising a combination of viral antigens that together may elicit a satisfactory level of protection. We present here a passive protection study of NS3.
We have produced hybridomas that secrete monoclonal antibodies directed against NS3 of dengue 1 virus (Hawaiian). Mice inoculated with these monoclonal antibodies showed an increase in survival times when challenged with a lethal dose of dengue 1 (Hawaiian) virus.

The production of monoclonal antibodies was based on the method described by Earley & Osterling (1985). Six-week old BALB/c mice were inoculated with a 20% (w/v) suspension of dengue 1 suckling mouse brain virus in phosphate-buffered saline (PBS) supplemented with 5% bovine serum albumin (BSA). Mice were inoculated with 0.1 ml virus intramuscularly and 0.5 ml subcutaneously on both days 1 and 3. On day 14, the mice were inoculated intraperitoneally with 0.5 ml of virus and with a final intravenous boost of 0.2 ml of virus on day 21. Three days later, the spleen was removed and the spleen cells were fused with P3U1 myeloma cells with 1 ml of 50% (w/v) polyethylene glycol 4000 (Merck) per 10^8 cells. The hybridoma cells were resuspended in Iscove’s modified Dulbecco medium (IMDM, Gibco) containing hypoxanthine, aminopterin and thymidine (HAT), and distributed into 96-well plates (Nunc).

The fused cells were put through a series of increasing-specific tests to select those producing monoclonal antibodies directed against NS3. Initially, positive clones were selected by testing the supernatants of hybridoma cultures for antibody secretion by ELISA. In the preparation of monoclonal antibodies, mouse brain virus was used as the immunogen but in the screening ELISA, the viral antigen was prepared in C6/36 mosquito cells in order to avoid selecting hybridomas secreting antibodies to mouse brain protein. For ELISA, microtitre plates were coated overnight at 4 °C with sonicated dengue 1 virus-infected C6/36 cell lysate. The five monoclonal antibodies did not inhibit haemagglutination by or fix complement with dengue 1 virus-infected cell lysate (lanes 1) but not from the control, uninfected cell lysate (lanes 2); monoclonal antibodies 7A4, 10D2 and 127F precipitated NS3 as well as another protein corresponding to NS5.

Monoclonal antibodies were tested using fluorescein isothiocyanate-labelled anti-mouse immunoglobulins (Zymed). The specificity of the monoclonal antibodies for dengue virus was established as fluorescent staining was observed only with infected cells and not with uninfected cells (data not shown).

The five monoclonal antibodies did not inhibit haemagglutination by or fix complement with dengue 1 and 2 mouse brain virus antigens, indicating that they were not directed against structural viral proteins. The haemagglutination inhibition (HI) test was performed as described by Clarke & Casals (1958) and the complement fixation test was based on the method of Hsiung & Fong (1982), both using sucrose–acetone-extracted suckling mouse brain virus antigens.

A radioimmunoprecipitation assay (RIPA) using Triton X-100 extracts of dengue 1 and 2 virus-infected...
Fig. 2. Western blot assay with monoclonal antibodies. The TrpE–NS3 fusion protein was generated by induction of the plasmid pATH10/TrpE-NS3(Eco-Eco)S with indoleacrylic acid (5 µg/ml). The bacteria were pelleted after 3 to 4 h, resuspended in cracking buffer (0.326 mM-NaH₂PO₄·H₂O, 12.68 mM-Na₂HPO₄, 14.54 mM-NaCl, 0.6 M-urea, 1% 2-mercaptoethanol, 1% SDS) and held at 37 °C for 1 to 2 h to dissolve the bacterial pellet. For PAGE and subsequent Western blotting onto nitrocellulose membranes, the bacterial extract was mixed with an equal volume of loading buffer and boiled before loading on a 10% polyacrylamide gel. The negative control (lane 1) was similarly prepared with the parent plasmid pATH10. Hybridoma tissue culture fluids were incubated for 1 h with nitrocellulose strips containing electroblotted proteins followed by washing in PBS-Tween and incubation with anti-mouse antibodies conjugated to horseradish peroxidase (Dako) for another h. Binding was visualized by addition of 4-chloronaphthol. All five monoclonal antibodies reacted similarly, with a thick band (arrow) from the TrpE-NS3 antigen preparation at approximately 67K. This band corresponded to the postulated Mr of the TrpE-NS3 fusion protein and was not observed in the strip with antigen prepared from pATH10.

C6/36 cells was used to identify the protein to which the monoclonal antibodies are directed. In RIPA, the five monoclonal antibodies precipitated proteins with the Mr of NS3 from the dengue virus-infected cell lysates; no proteins were precipitated from uninfected cell lysates (Fig. 1). Three monoclonal antibodies (7A4, 10D2 and 127F) also coprecipitated a protein of Mr corresponding to NS5 which together with NS3 has been postulated to be a replicase component (Westaway, 1977; Rice et al., 1985; MacKow et al., 1987). This coprecipitation could be due to NS3 and NS5 existing as a heterodimer.

It is unlikely that the monoclonal antibodies are not directed against NS3 but are against NS5 as they reacted with a TrpE–NS3 fusion protein expressed from the plasmid pATH10/TrpE-NS3(Eco-Eco)S (the parent plasmid pATH10 was provided by Dieckmann & Tzagoloff, 1985). This NS3 expression plasmid was constructed by inserting an EcoRI fragment from nucleotide positions 5555 to 6348 of the NS3 gene into the EcoRI site of pATH10 which had an artificial stop codon generated by digestion of the construct with BamHI followed by filling in with the Klenow fragment of DNA polymerase I and ligation. Our data, however, do not exclude the possibility that NS3 and NS5 share epitopes that were recognized by the monoclonal antibodies, resulting in coprecipitation. Western blot analysis with the TrpE–NS3 fusion protein confirmed that the five monoclonal antibodies were reactive with NS3 (Fig. 2).

In protection studies with these monoclonal antibodies, six to 12 Swiss albino mice (13- to 14-day old) were each inoculated intraperitoneally with 0.2 ml of tissue culture fluid containing ammonium sulphate-precipitated antibody (400 or 800 µg). Two control groups were included, one inoculated with PBS and the other with BSA. The mice were challenged intracerebrally 24 h later with 0.1 ml of 100 LD₅₀ of dengue 1 virus. The LD₅₀ titre of the challenge virus was determined for mice of the same age group by the method of Reed & Muench (1938).

The survival index Y of each group of mice was calculated from the method of Smith & Westgarth (1957) as follows:

\[
Y = (1000 \div k) \sum_{i=1}^{k} \frac{1}{t_i}
\]

where \( k \) is the number of mice in each group and \( t_i \) is the number of days survived by mouse no. \( i \).

The protective capacity of the monoclonal antibody is correlated with an increase in survival time of mice immunized with monoclonal antibody relative to mice receiving only PBS when challenged with a lethal dose of dengue 1 virus. The results of the protection study is presented in Table 1. The protective capacity of the monoclonal antibodies increased with increasing amount of the antibody (Table 2). When 400 µg was given, only three monoclonal antibodies (7A4, 7B3 and 123E) conferred equivocal protection whereas neither 127F nor 10D2 was protective. However, at a dose of 800 µg, all five monoclonal antibodies resulted in Y values greater than \( Y_L \) but less than \( Y_K \), indicating
Table 1. Numbers of surviving mice at various days after lethal challenge with dengue 1 virus

<table>
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<th>Monoclonal antibody</th>
<th>Amount of protein (µg)</th>
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<th>8</th>
<th>9</th>
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* Between days 2 to 4 and 16 to 20 there were no changes in the numbers of surviving mice.

Table 2. Protective capacity of monoclonal antibodies directed against non-structural protein NS3 of dengue 1 virus

<table>
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<th>Monoclonal antibody</th>
<th>Y (survival index)</th>
<th>Protection status</th>
<th>P value</th>
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<td>400 µg</td>
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<td>95.13</td>
<td>Equivocal</td>
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<td>108.07</td>
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<td>Equivocal</td>
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<td>123E</td>
<td>123.21</td>
<td>107.36</td>
<td>Equivocal</td>
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<tr>
<td>127F</td>
<td>150.79</td>
<td>88.10</td>
<td>Not protective</td>
</tr>
<tr>
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<td>Not protective</td>
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<tr>
<td>PBS</td>
<td>146.83</td>
<td>130.04</td>
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<tr>
<td>BSA</td>
<td>Not done</td>
<td>136.9</td>
<td>Not done</td>
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</table>

* Amount of monoclonal antibody preparation inoculated expressed as µg of protein. Monoclonal antibodies were regarded as protective if the Y value was lower than YL (51.26) and equivocal if the Y value was greater than YL but lower than Ys (112.71). A Y value greater than Ys would be regarded as not protective.

equivocal protection. For these five monoclonal antibodies, the protein concentration of 800 µg was the better concentration for demonstrating protective capacity. Though the protection status for 7A4, 7B3, 123E and 10D2 was equivocal, the decrease in Y was significant (P ≤ 0.05) by Student's t-test. 127F, though equivocal, did not result in a significant decrease in the survival index Y.

To show that the demonstrated decrease in the survival index with the monoclonal antibodies is specific, several controls were included. All mice inoculated with PBS, the diluent for the monoclonal antibodies, died. The survival indices of the mice inoculated with BSA, which was included to determine whether a reduction in survival index could be obtained with a non-specific protein, did not differ from those for mice receiving PBS. In addition, a pooled dengue HI-positive polyclonal human serum showed equivocal protection with a significant increase in survival time when compared with PBS (P < 0.01). The observed increase in survival time of challenged mice was, therefore, specific and significant.

Antibodies to NS3 may play a major role in the immune response to dengue virus infection. Preugschat & Strauss (1989), studying the processing of the precursor polyprotein translated from the dengue virus genome, suggest that NS3 might be an autoprotease cleaving itself and another non-structural protein, NS2a, from the polyprotein. Kurane et al. (1989) reported that NS3 has strong T cell-activating epitopes. These data, together with the postulate that NS3 is a replicase component (Westaway, 1977; Rice et al., 1985; MacKow et al., 1987), indicate that antibodies to NS3 may interfere with processing of the polyprotein and activation of the T cell response. We observed as well that the five monoclonal antibodies produced are cross-reactive as they reacted in IFA and RIPA to both dengue 1 and dengue 2 viral antigens. In addition, NS3 is antigenic in man as antibodies to NS3 have been detected in dengue patients' sera by Western blot analysis (unpublished results). NS3
may thus have a role in conferring immunity to dengue virus infection and has potential as a component of a candidate subunit vaccine.

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References


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