An Immunoprotective Monoclonal Antibody Directed against
Leptospira interrogans serovar copenhageni

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A monoclonal antibody (mAb) was prepared by hybridoma technology in BALB/c mice
immunized to Leptospira interrogans serovar copenhageni. This mAb agglutinated serovars

céphageni and icterohaemorrhagiae to high titres and protected hamsters, dogs and monkeys
against challenge with a virulent strain of serovar copenhageni. The mAb gave protection to
hamsters at dilutions up to 1 in 1000; at a 1 in 10 dilution the protective effect lasted for at least
two weeks. Biochemical analysis by SDS-PAGE and Western blotting indicated that this mAb
reacted with an epitope of a carbohydrate nature.

INTRODUCTION

Leptospiral vaccines are used extensively in domestic animals and effectively prevent clinical
disease (Bey et al., 1974; Stoener, 1976; Broughton & Scarnell, 1985). However, vaccination
with whole killed leptospira has disadvantages, including the occurrence of side-effects and the
need to revaccinate to maintain immunity (Babudieri et al., 1973; Torten et al., 1973; Chen Ting
Zuo, 1986). Although the immune response to infection with leptospires is well-known, the
nature of the antigens giving protection is still unclear. Monoclonal antibodies (mAbs) are
powerful tools which can be used for antigen analysis, classification and immunoprotection. An
mAb reacting with a carbohydrate determinant in the lipopolysaccharide (LPS) passively
protected against experimental infection with the homologous Leptospira serovar (Jost et al.,
1986). Antibodies against LPS from other Gram-negative organisms, as well as mAbs directed
against polypeptides, have also been used to protect against experimental infection (Pennington
& Kuchmy, 1980; Boyle et al., 1982; Sawada et al., 1984). We have used mAbs for the
classification of many different Leptospira serovars (Terpstra et al., 1985, 1987). One of several
mAbs used for classification of the Icterohaemorrhagiae serogroup proved to be strongly
protective. We report here on the passive protection of hamsters, dogs and monkeys against
challenge with a virulent Leptospira strain, provided by an mAb specifically directed to serovars
icterohaemorrhagiae and copenhageni.

METHODS

Production of mAbs. BALB/c mice were immunized against Leptospira interrogans serovar copenhageni.
Immunization and fusion were done according to standard techniques (Köhler & Milstein, 1975) as described by
Terpstra et al. (1985). Clones were selected by the Leptospira microscopic agglutination test (MAT). The

Abbreviations: mAb, monoclonal antibody; LPS, lipopolysaccharide; MAT, microscopic agglutination test;
i.p., intraperitoneal(ly); i.v., intravenous(ly); PM, post-mortem; GMRT, geometric mean reciprocal titre.
immunoglobulin class of the selected mAb was determined using specific goat antisera (Nordic Diagnostics) by double gel diffusion.

*Bacteria.* The *Leptospira* strains used in this study are reference strains from our collection, with the exception of the challenge strain of *Leptospira interrogans* serovar *copenhageni* which was originally provided by Duphar (The Netherlands).

**Passive protection experiments.** Five groups of six 6-week-old Syrian hamsters were injected intraperitoneally (i.p.) with a serial tenfold dilution (1 in 10 to 1 in 100000) of 0.5 ml mAb coded F12 C3. Six hamsters were given sterile phosphate-buffered saline (PBS) (20 mM-Na₂HPO₄, 150 mM-NaCl, adjusted to pH 7.4 with HCl) as a control. All 36 hamsters were challenged 1 d after immunization by the subcutaneous injection of 10⁸ leptospires of the virulent strain of serovar *copenhageni*. A further five groups of six hamsters each were immunized i.p. with 0.5 ml mAb F12 C3 at a 1 in 10 dilution and challenged 1, 2, 4, 8 and 16 weeks after immunization.

Stray dogs of varying age and breed were collected by the Animal Control Center, Barbados. Seven of these animals which were seronegative for 15 pathogenic *Leptospira* serogroups were used in this study. Three were injected intravenously (i.v.) with 1 ml mAb F12 C3 and four control dogs received sterile PBS as a placebo. All seven dogs were challenged 1 d later by the i.p. injection of a washed suspension of 2 × 10⁸ virulent *Leptospira* serovar *copenhageni*.

Feral vervet monkeys (*Cercopithecus aethiops sabaeus*) were held captive at the Barbados Primate Research Center and Wildlife Reserve. Eight of these animals which were seronegative for 15 pathogenic *Leptospira* serogroups were selected. Four monkeys were immunized by the i.v. injection of 1 ml mAb F12 C3; the second group of four monkeys (a control) received sterile PBS. After 1 d, all eight monkeys were challenged by the i.p. injection of 0.1 ml of a washed suspension containing 10⁸ virulent leptospires of serovar *copenhageni*.

**Isolation procedures.** Cultures of samples from hamsters were not attempted because at death all of the hamsters showed clinical signs of a severe leptospiral infection (Broughton & Scarnell, 1985).

Samples of blood and urine were collected from dogs six times after challenge, and post-mortem (PM) specimens of kidney and liver on day 15. Urine was collected in sterile plastic bags after first injecting the animals with the appropriate dosage of Lasix 10 mg ml⁻¹ (Hoechst). On each occasion five drops of blood were inoculated into 5 ml polysorbate medium (EMJH) as described by Ellinghamaus & McCullough (1965) and modified by Johnson & Harris (1967). The cultures were further enriched with 1% (v/v) rabbit serum. Five drops of urine were inoculated into 5 ml EMJH medium to which was added 5-fluorouracil (200–300 μg ml⁻¹). Another five drops of urine were filtered through a 0.45 μm disposable filter unit (Millex Millipore) and inoculated into another vial containing only 5 ml EMJH medium. At PM examination approximately 0.1 g tissue was removed aseptically from each of the liver and kidney and inoculated into 5 ml EMJH medium.

Monkey blood for culture was collected after 3, 7 and 15 d. Five drops were inoculated in 5 ml EMJH medium, as above.

**Characterization of mAb F12 C3.** Antigens were prepared by sonication of the residual pellet of a centrifuged culture of leptospires. One part of the sonicate was treated with pronase for digestion of proteins, while another part was treated with periodate for cleavage of glucoside ring structures, as described by Vennegoor et al. (1985).

Prior to electrophoresis, antigens were treated with 5% (v/v) 2-mercaptoethanol and 2% (w/v) SDS at 100 °C for 5 min.

Gels were stained for proteins with Coomassie Brilliant Blue and for glycoconjugates by a silver stain (Tsai & Frasch, 1982).

**SDS-PAGE.** Sonicated antigens of serovar *Brilleni* were subjected to SDS-PAGE using the discontinuous buffer system of Laemmlli (1970).

**Western blot analysis.** Sonicated preparations were subjected to SDS-PAGE and the separated bands were transferred electrophoretically (25 V at room temperature for 18 h) to nitrocellulose (0.45 μm, Schleicher and Schüll) in a Bio-Rad 'Transblot' apparatus using 25 mM-Tris buffer containing 192 mM-glycine and 20% (v/v) methanol, pH 8.3. After electrophoretic transfer, the nitrocellulose sheets were washed for 30 min with PBS containing 0.5% (v/v) Tween 20 (PBST). mAbs (ascitic fluid diluted 1 in 1000) in 10% (v/v) foetal calf serum (FCS) in PBST were added to the membranes, which were then incubated with gentle agitation for 1 h at room temperature. The membranes were then washed five times (10 min each wash) with PBST and incubated with secondary antibody [1 in 1000 dilution of goat anti-mouse IgG-horseradish peroxidase conjugate (Pasteur)] in PBST containing 10% (v/v) FCS for 1 h at room temperature with gentle agitation. The membranes were further washed five times with PBST and immersed in a substrate solution of 10 ml 0.1 M-acetate/citrate buffer, pH 6.0, to which was added 100 μl tetramethylbenzidine (TMB) (Sigma) in dimethylsulphoxide (10 mg ml⁻¹), 100 μl diocetyl sodium sulphosuccinate (DOSS) (Sigma) in dimethylsulphoxide (33 mg ml⁻¹) and 100 μl 0.5% (v/v) H₂O₂. After 5 min the blot was allowed to dry.

**Agglutination absorption.** Serial twofold dilutions of mAb F12 C3 starting at 1 in 100 were incubated for 2 h at 37 °C with 50 μg sonicate and 50 μg pronase-treated sonicate of serovar *copenhageni*. After incubation, live leptospires of the homologous serovar were added. The MAT was read 2 h later.
Table 1. Challenge of hamsters with virulent copenhageni leptospires 1 d after passive immunization with mAb F12 C3

<table>
<thead>
<tr>
<th>Dilution of mAb</th>
<th>Proportion of hamsters surviving 14 d after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 in 10</td>
<td>6/6</td>
</tr>
<tr>
<td>1 in 100</td>
<td>6/6</td>
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<tr>
<td>1 in 1000</td>
<td>6/6</td>
</tr>
<tr>
<td>1 in 10000</td>
<td>0/6</td>
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<tr>
<td>1 in 100000</td>
<td>0/6</td>
</tr>
<tr>
<td>PBS control</td>
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Table 2. Challenge of hamsters with virulent leptospires 1–16 weeks after passive immunization with mAb F12 C3 (1 in 10)

<table>
<thead>
<tr>
<th>Time after immunization (weeks)</th>
<th>Proportion of hamsters surviving 14 d after challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6/6</td>
</tr>
<tr>
<td>2</td>
<td>6/6</td>
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<td>4</td>
<td>4/6</td>
</tr>
<tr>
<td>8</td>
<td>4/6</td>
</tr>
<tr>
<td>16</td>
<td>0/6</td>
</tr>
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</table>

Table 3. Leptospira microscopic agglutination titres before and after immunization and challenge of dogs using Leptospira interrogans serovar copenhageni as antigen

<table>
<thead>
<tr>
<th>Time after immunization (d)</th>
<th>GMRT*</th>
<th>Immunized dogs</th>
<th>Control dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>63</td>
<td>1521</td>
<td></td>
</tr>
<tr>
<td>10</td>
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<td>6085</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>6085</td>
<td></td>
</tr>
</tbody>
</table>

* The GMRT was determined for three immunized dogs and four control dogs.

RESULTS

Preliminary immunization experiments with different mAbs of the Icterohaemorrhagiae group in hamsters showed that mAb F12 C3 was the most promising. This IgG 2a class mAb gave protection against challenge with virulent serovar copenhageni. Immunization with a 1 in 1000 dilution of mAb F12 C3 effectively protected hamsters when challenged the next day (Table 1). Hamsters challenged 1–16 weeks after immunization with a 1 in 10 dilution of this mAb were fully protected for at least two weeks. No protection was observed 16 weeks after immunization (Table 2). Although our serovar copenhageni was highly virulent for hamsters, the Barbadian dogs showed no clinical symptoms of infection when challenged with the same strain. However, leptospires were isolated from all four unprotected control dogs: from four blood samples 1 d after challenge, from three blood samples 2 d after challenge, from one urine sample 2 d after challenge, and from two liver samples and one kidney sample after PM.

Leptospires could not be isolated from immunized dogs. This observation can be correlated with the MAT titres that were found in both immunized and control dogs (Table 3). The passively immunized dogs showed a slight but negligible rise with a maximum geometric mean reciprocal titre (GMRT) of 1 in 63 which was most likely caused by the immunization with the
mAb. The control group showed seroconversion to a peak GMRT value of 1 in 6085. The monkeys showed a similar MAT pattern (Table 4) where a slight rise in titre was observed after immunization. The control group clearly showed seroconversion. The highest GMRT recorded was 1 in 1279. In the passively immunized monkeys low positive MAT titres persisted for several months. No leptospires could be isolated from the monkeys.

Absorption of mAb F12 C3 with sonicate and pronase-treated sonicate of serovar copenhageni in both samples showed a 16-fold fall in titre indicating that the antigen was not a protein. The nature of the antigen recognized by mAb F12 C3 was further studied in SDS-PAGE followed by Western blotting (Fig. 1). The antigen recognized by mAb F12 C3 appeared as a broad diffuse band between 24 and 40 kDa (lanes 3), which could only be stained with silver (lanes 2). Peptide digestion by pronase (Fig. 1b) did not affect antibody binding, thus excluding the association of the binding site with protein. After cleavage of glucoside ring structures by periodate (Fig. 1c) the antigen could not be visualized in Western blot. These results all suggest that the antigen recognized by mAb F12 C3 is a glycoconjugate.

Table 4. Leptospira microscopic agglutination titres before and after immunization and challenge of monkeys using Leptospira interrogans serovar copenhageni as antigen

<table>
<thead>
<tr>
<th>Time after immunization (d)</th>
<th>GMRT*</th>
<th>Immunized monkeys</th>
<th>Control monkeys</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>113</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>113</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>95</td>
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</tr>
<tr>
<td>160</td>
<td>48</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>

* The GMRT was determined for four immunized monkeys and four control monkeys.

Fig. 1. Characterization of antigens from Leptospira interrogans serovar copenhageni, recognized by mAb F12 C3. (a) Sonicated antigen, (b) sonicated antigen digested with pronase, (c) sonicated antigen treated with periodate. Lanes 1, SDS-PAGE profile of antigen stained with Coomassie Brilliant Blue for proteins; lanes 2, SDS-PAGE profile of antigen stained with silver for glycoconjugates; lanes 3, Western blot profile of mAb F12 C3.
DISCUSSION

In six series of fusions a panel of mAbs was prepared which enabled the classification of the serovars of the Icterohaemorrhagiae group (Korver et al., 1988). One of the first mAbs produced reacted strongly in the agglutination test with serovars icterohaemorrhagiae and copenhageni which are probably (worldwide) the most important serovars belonging to the Icterohaemorrhagiae group. This mAb, which was coded F12 C3, was investigated for possible immunoprotection against challenge with virulent leptospires. The mAb was administered in different doses to hamsters, dogs and monkeys.

Immunized hamsters were protected even with mAb dilutions of 1 in 1000 and protection persisted for at least 2 weeks after immunization with mAb F12 C3 diluted 1 in 10. However, these hamsters were infected with very high numbers of leptospiral organisms, so that, when naturally infected, protection could last longer than 2–8 weeks. Although the control dogs and monkeys showed no symptoms of clinical leptospirosis after challenge with virulent serovar copenhageni leptospires, the lack of seroconversion in the immunized animals showed that the mAb was itself protective. Moreover, leptospires could not be isolated from these immunized dogs.

It is interesting to note that an mAb directed against the LPS of Gram-negative bacteria promoted phagocytosis and could be used in the immunotherapy of Gram-negative bacterial sepsis (Dunn et al., 1986). Passive immunization with an mAb directed against leptospiral LPS showed the powerful protective effect of phagocytosis as a defence mechanism against infection with leptospires in mice (Isogai et al., 1986). It is conceivable that the protection given by the mAb was caused by a similar mechanism.

Biochemical characterization of the antigen recognized by mAb F12 C3 indicated that the epitope is a carbohydrate, probably part of a polysaccharide. Silver staining revealed that the major band was only present in the sonicate and pronase-treated sonicate. Jost et al. (1986) reported the production of an mAb directed against a carbohydrate determinant in the LPS of the same Leptospira serovar. This broadly reactive mAb gave protection in guinea pigs but was weak in comparison with mAb F12 C3.

The antibodies produced in response to natural and experimentally induced leptospiral infections are agglutinins, suggesting that such antibodies are important in immunity towards risis disease (Adler & Faine, 1978). All the agglutinating mAbs prepared and characterized in our laboratory appear to be directed towards polysaccharide antigens (Terpstra et al., 1985, 1987). Ono et al. (1984) demonstrated strongly immunogenic epitopes consisting of a mixture of two oligosaccharides located on the outer membrane using an mAb directed against serovar canicola.

We conclude that mAbs have a wide range of possible applications in the study of leptospirosis, including the isolation and purification of polysaccharide antigens which may eventually be used in vaccines. At the same time, characterization of the antigenic determinant involved in the development of protective immunity is necessary for the production of such vaccines. In the past, serotherapy with polyvalent anti-leptospira antiserum was applied with some success but later abandoned in favour of antibiotic therapy (van Thiel, 1948). Serotherapy as an adjunct to antibiotics has been reappraised in the treatment of Gram-negative septicemia to control septic shock, which may develop despite the administration of efficacious antibiotics (Zeigler et al., 1982; Adhikari et al., 1985; Baumgartner et al., 1985). Antiserum to bacterial LPS is thought to detoxify endotoxin which is probably the main cause of septic shock. Although the mechanism of pathogenicity in leptospirosis is not fully known, it is certain that endotoxins play an important role (Adler et al., 1980). In this context, the influence of mAbs on the course of disease in leptospirosis and the duration of protection against natural infection deserves more attention.

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REFERENCES


