Characterization and protective activity of a monoclonal antibody against a capsular epitope shared by *Streptococcus suis* serotypes 1, 2 and 1/2

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A monoclonal antibody (mAb Z3) was produced using BALB/c mice immunized with whole cells of *Streptococcus suis* serotype 2 reference strain S735. Screening by dot-ELISA showed that mAb Z3, of isotype IgG2b, reacted only with reference strains and field isolates of *S. suis* serotypes 1, 2 and 1/2. The recognized epitope was demonstrated to be polysaccharide in nature by periodate oxidation, and located in the capsule, since mAb Z3 reacted with purified capsular material by immunoblotting and was able to stabilize the capsule as shown by electron microscopy. Further characterization indicated that mAb Z3 may react specifically with the sialic acid moiety of the capsule, a common constituent of the polysaccharidic capsular material of the three capsular types, since sialidase-treated cells did not react with mAb Z3 in immunoblotting or indirect ELISA. Purified mAb Z3 was shown to significantly increase the rate of phagocytosis of *S. suis* cells by porcine monocytes and to activate the clearance of bacteria from the circulation in experimentally infected mice. However, mAb Z3 only offered partial protection to mice challenged with a minimal lethal dose. Thus, even though the capsule of *S. suis* seems to be an important virulence factor, the epitope recognized by mAb Z3 does not appear to be involved in complete protection against infection.

**Keywords:** *Streptococcus suis*, capsule, monoclonal antibody, protection, clearance

**INTRODUCTION**

*Streptococcus suis* is a worldwide cause of meningitis, arthritis, endocarditis and septicemia in swine (Higgins et al., 1992). It is also recognized as an important human pathogen (Arends & Zanen, 1988). Of the 35 serotypes of *S. suis*, serotype 2 is the most prevalent in diseased animals (Higgins et al., 1992). The pathogenesis of the different types of infections due to this bacterium is still unclear. Of the many cell components and toxic products of *S. suis* that have been suggested as virulence factors (Gottschalk et al., 1995; Jacques et al., 1990; Vecht et al., 1989) the polysaccharide capsule appears to be one of the most important (Brazeau et al., 1996; Elliott & Tai, 1978; Gottschalk et al., 1992). Although the capsular material possesses antiphagocytic properties (Brazeau et al., 1996), it has been demonstrated that both virulent and avirulent encapsulated strains belonging to serotype 2 are ingested by phagocytes in the absence of complement and specific antibodies (Brazeau et al., 1996; Williams, 1990). Serotype 2 capsular material contains sialic acid, an important virulence attribute for some other bacteria (Smith, 1993; Wessels et al., 1989). However, *S. suis* sialic acid did not appear as a critical virulence factor (Charland et al., 1996).

Monoclonal antibodies (mAbs) are useful tools for the study of bacterial virulence factors. For bacteria such as group B streptococci, *Neisseria meningitidis* and *Escherichia coli*, mAbs against their capsular material have been shown to induce good protection (Cross et al., 1983; Egan et al., 1983; Hurpin et al., 1992). The aim of this paper is to report the production, characterization and evaluation of the protective activity of a murine mAb that reacts specifically with the capsular polysaccharides of *S. suis* serotype 2.

**Abbreviations:** i.p., intraperitoneal(y); SNA, *Sambus nigra* agglutinin.
METHODS

Bacterial strains and culture conditions. The *S. suis* serotype 2 reference strain S735 was kindly provided by Dr J. Henrichsen (Statens Seruminstitut, Copenhagen, Denmark). The remaining 34 serotype reference strains came from our collection. A total of 484 *S. suis* field isolates were kindly provided by Dr R. Higgins (Clinical Bacteriology Laboratory, Faculty of Veterinary Medicine, University of Montreal). Of these, 15, 67 and 142 isolates belonged to serotypes 1, 1/2 and 2, respectively. Other isolates represented the remaining 32 serotypes. These isolates had previously been serotyped by standard techniques (Higgins & Gottschalk, 1990). Culture methods and media have been described previously (Charland et al., 1996).

Monoclonal antibody production. BALB/c mice were injected i.p. at 2-week intervals with a 0.3% formalinized whole-cell S735-Quil A saponin suspension (25 μg per mouse; Superfos Biosector, DK-Vedbaek, Denmark). A total of five injections was required to obtain a high titre of antibodies in an indirect ELISA using a whole-cell antigen (Del Campo Sepulveda et al., 1996). Three days after the booster inoculation, mice were serologically tested with the indirect ELISA and the spleen of the mouse showing the highest reaction was recovered and fused with myeloma cell line SP2/0 using 50% (w/v) PEG 1500 (Sigma) as a fusogen (Köhler & Milstein, 1975). Hybridoma supernatants were screened for the presence of *S. suis* type 2 antibodies using the indirect ELISA (Del Campo Sepulveda et al., 1996). Positive hybridomas were then tested with the same method, but with purified capsular polysaccharide material as an antigen (Del Campo Sepulveda et al., 1996). When positive, they were cloned by the limiting dilution method (Kamata et al., 1985). The immunoglobulin class of mAb was determined using the Sigma ImmunoType Mouse Monoclonal Antibody Isotyping Kit. Ascitic fluid was produced by i.p. injection of BALB/c mice primed with pristane with hybridomas.

ELISA tests. A dot-ELISA was performed with bacteria grown overnight on bovine blood agar plates at 37 °C and from which single colonies were used as inocula for Todd–Hewitt broth (THB, Difco). Suspensions of each isolate adjusted to an OD₅₅₀ of 1 ± 0.2 were spotted onto a nitrocellulose membrane and the dot-ELISA was carried out as described by Belanger et al. (1992). An ELISA-competition test with a sialic-acid-binding lectin from *Sambucus nigra*, SNA I, which is known to react with the sialic acid moiety of *S. suis* type 2 capsule (Charland et al., 1995), was also carried out. Ascitic fluid, produced with clone Z3, diluted to yield an A₄₅₀ of approximately 1.0 in indirect ELISA was mixed with increasing amounts of SNA I and the solutions added to purified capsular material coated to microplates and reactions revealed as described by Del Campo Sepulveda et al. (1996). In addition, an inhibition-ELISA was performed. Ascitic fluid, prepared as described above, was mixed with untreated, or with sialidase-, periodate- or proteinase K- (500 μg proteinase K ml⁻¹, 55 °C, 1 h; Sigma) treated whole-cell antigen of type 2 reference strain S735, whole-cell antigens of types 1 or 1/2 reference strains or purified sialic acid (Sigma), respectively. Suspensions were incubated at room temperature for 15 min, centrifuged and supernatants added to purified capsular material coated to microplates. The percentage inhibition was calculated using the formula 100(1−A/B), where A and B are A₄₅₀ in the presence and absence of inhibitor, respectively.

PAGE and immunoblotting analysis. PAGE (8%, w/v, separating gel) and blotting on PVDF-N membrane (Millipore) of untreated or periodate-treated (NaIO₄, 20 mM, 25 °C, 1 h) purified capsular material of *S. suis* type 2 were performed using an improved technique for carbohydrates as described by Tikkonen et al. (1995). SDS-PAGE (8%, separating gel) and blotting on a nitrocellulose membrane (Bio-Rad) of untreated or sialidase-treated (0.6 U sialidase from *Clostridium perfringens* ml⁻¹ acetate buffer, pH 5.5, 37 °C, 3 h, Boehringer Mannheim) whole-cell antigen of *S. suis* serotype 2 were performed as described by Radacovici et al. (1992). Monoclonal antibody Z3 reaction on both blots was revealed as described by Radacovici et al. (1992).

Sialic acid determination. Sialic acid concentrations were determined using the Warren–Aminoff method (Warren, 1963).

Transmission electron microscopy. Transmission electron microscopy after immunostabilization with mAb Z3 was carried out as previously described (Jacques et al., 1990). Bacterial suspensions were adjusted to OD₅₅₀ 1.8 and exposed to ascitic fluid diluted 1:1 for 1 h at 4 °C. Bacterial cells were then suspended in 0.1 M cadycyde buffer, pH 7.0, containing 5% (v/v) glutaraldehyde and 0.15% ruthenium red. Fixation was for 2 h at 20 °C. Cells were then immobilized in 4% (w/v) agar, washed five times in cadycyde buffer containing 0.05% ruthenium red, and postfixed with 2% osmium tetroxide for 2 h. Samples were washed as above and dehydrated in a graded series of acetone washes containing 0.05% ruthenium red. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV.

Oposonization and phagocytic assay. Immunoglobulins were first purified by chromatography on a protein G–Sepharose column (Pharmacia), with 3 M NaCl, 1.5 M glycine, pH 8.9, as binding buffer, and 0.1 M citric acid, pH 4.0, as elution buffer. Purified IgG was then dialysed against 0.1 M PBS (14 mM NaCl, 0.3 mM KCl, 1 mM Na₂HPO₄, 0.15 mM KH₂PO₄, pH 7.4) overnight and lyophilized. Immunoglobulin concentration was determined by the method of Markwell et al. (1978). The opsonizing capacity of mAb Z3 was determined using a porcine blood monocytes phagocytic assay (Charland et al., 1996). Strain S735 was opsonized with heat-inactivated porcine serum (to prevent opsonization by complement; negative control), with 80 μg purified mAb Z3 or with polyclonal anti-type 2 rabbit serum as a positive control. Phagocytosis was left to proceed for 3 h at 37 °C, 5% CO₂. Cells were then stained with the vital dye acridine orange, counterstained with crystal violet to quench monocyte external fluorescence, and percentage phagocytosis was measured under UV light. The assay was repeated in three independent experiments.

Protection assay. The protective capacity of mAb Z3 was tested in a standardized mouse model of infection (Beaudoin et al., 1992). The test was repeated in three independent experiments. Strain S735 grown for 6 h in THB containing 10% foetal bovine serum (FBS) was adjusted to a concentration of 10⁸ c.f.u. ml⁻¹ in THB-FBS, which represents the minimal lethal dose to kill all control mice (Keleka et al., 1990). Bacterial suspensions were mixed with 0.8 mg of purified mAb Z3 ml⁻¹ and 1 ml per mouse was injected intraperitoneally into a group of 15 BALB/c mice, so that mice received the equivalent of 40 mg mAb Z3 kg⁻¹. Another group of 15 mice received only the bacterial suspension as a negative control whereas the final group received bacteria mixed with a polyclonal anti-type 2 rabbit serum diluted as already described (Gottschalk et al., 1992) as a positive control. When
a dose of $10^7$ c.f.u. ml$^{-1}$ was used as a challenge, one group of mice received isotype-matched, non-specific monoclonal antibody S1 G8 F10 as a negative control, a mAb that reacts with *Actinobacillus pleuropneumoniae* type 1 lipopolysaccharides (Lairini *et al.*, 1995). Mortality and clinical signs were then monitored twice a day for a week.

**Bacterial clearance test.** A bacterial clearance test was performed with mAb Z3 and repeated in two independent experiments. Bacteria were grown in THB-FBS as above and the concentration adjusted to $3 \times 10^8$ c.f.u. ml$^{-1}$ to keep all mice alive and slightly affected by infection. One group of six mice was injected i.p. with 1 ml S735 suspension, another group with S735 mixed with mAb Z3 (40 mg kg$^{-1}$) and a last group with S735 mixed with mAb S1 G8 F10 (40 mg kg$^{-1}$) as a negative control. Blood samples were taken in duplicate from the orbital artery at various time intervals after injection and viable counts were performed on blood agar plates.

**RESULTS AND DISCUSSION**

**mAb Z3 and its capsular type specificity**

The screening steps of mAb production led to the selection of positive clone Z3, producing immunoglobulins of class IgG2b. The capsular type specificity of mAb Z3 was controlled against reference strains of all *S. suis* serotypes using a dot-ELISA. Of the 35 *S. suis* reference strains, only serotypes 1, 2 and 1/2 reacted with mAb Z3. Despite the fact that the epitope recognized by mAb Z3 probably involves a sialic acid moiety (see below), reference strains of other sialic-acid-containing serotypes (Charland *et al.*, 1995) did not react with the antibody. It is possible that different serotypes contain sialic acid in their respective capsular material, yet the specific epitope recognized by mAb Z3 is only present in serotypes 1, 2 and 1/2. To confirm the specificity of mAb Z3, attempts were made to detect this epitope on a total of 484 field isolates representing the 35 serotypes of *S. suis*. Results showed that 93%, 98% and 96% of isolates, belonging to serotypes 1, 2 and 1/2 respectively, were positive. Isolates from all other serotypes were negative. It is conceivable that the few negative field strains which belong to serotypes 1, 2 or 1/2 were low-encapsulated strains. In fact, serotyping is unknown, removal of other epitopes by sialidase treatment cannot be ruled out.

**Characterization of the epitope recognized by mAb Z3**

Results from immunoblots showed that mAb Z3 reacted with the type 2 capsule in the high-molecular-mass region. Similar results were obtained by Del Campo Sepulveda *et al.* (1996) and Tikkanen *et al.* (1995) using polyclonal antibodies. It has been reported that the molecular mass of the *S. suis* serotype 2 capsule is about 310 kDa (Katsumi *et al.*, 1996). The epitope was shown to be polysaccharide in nature, since the reaction was affected by periodate oxidation (Fig. 1, lane B). In addition, proteins did not appear to be involved in the reaction since the blotting method of Tikkanen *et al.* (1995), which uses a nitrocellulose membrane between the gel and the PVDF-N membrane to prevent transfer of interfering proteins, allowed a clear reaction between mAb Z3 and capsular material. While mAb Z3 gave similar results with untreated whole-cell antigen of type 2 (Fig. 1, lane C), sialidase treatment completely eliminated the reaction (Fig. 1, lane D). Similar results were obtained with whole-cell antigens of capsular types 1 and 1/2 (data not shown). This indicates that the sialic acid moiety of the capsule may be involved in the recognition of the epitope by mAb Z3. Our results are in agreement with those of Katsumi *et al.* (1996), who reported that, after complete removal of capsular sialic acid, reactivity with anti-type 2 serum was no longer present. They also suggested that the sialic acid moiety contains the reactive epitope in the capsule. However, since the complete structure of the *S. suis* capsule is unknown, removal of other epitopes by sialidase treatment cannot be ruled out.

The ELISA-competition test showed a competitive
reaction between the SNA I lectin and mAb Z3 (Fig. 2). This is further evidence for the sialic acid nature of the recognized epitope, although the possibility of competition by steric hindrance cannot be excluded.

An inhibition-ELISA was developed to ascertain which antigens could inhibit the reaction between capsular material and mAb Z3. Fig. 3 shows that the reaction obtained in the indirect ELISA between mAb Z3 and purified capsule can be strongly inhibited by untreated or proteinase K-treated but not by periodate- or sialidase-treated serotype 2 whole-cell antigen. Serotype 1/2 whole-cell antigen presented an inhibition activity similar to that obtained with type 2 antigen. Interestingly, serotype 1 whole-cell antigen presented a low inhibition potential (25–30%). This could be explained, in part, by a lower amount of capsular material and/or capsular sialic acid. In fact, sialic acid concentrations of the reference strains of S. suis serotypes 1, 1/2 and 2 were shown to be 0.44±0.22 μg mg⁻¹, 1.57±0.22 μg mg⁻¹ and 3.36±0.22 μg mg⁻¹ respectively (means±sd, n = 3). In addition, the agglutination reaction obtained between the type 1 strain and SNA I lectin was significantly weaker than those obtained with types 2 and 1/2 (Charland et al., 1995). Capsular material of type 1 was four times thinner than that of the two other types (Jacques et al., 1990). Sialic acid or capsular material concentration may have been high enough to obtain a positive reaction by dot-ELISA but not quite high enough to have a significant inhibition potential on mAb Z3. On the other hand, a weak affinity of mAb Z3 for type 1 capsule coupled to amounts of capsule above saturation could also be responsible for the results obtained in this study. Although sialic acid did not present any inhibition potential of mAb Z3 (data not shown), the fact that the sialic acid moiety of the capsular material is involved in the epitope recognized by the mAb is still plausible. Indeed, high numbers of monomers are needed for functional binding to other sialic-acid-specific mAbs (Hayrinen et al., 1989; Hurpin et al., 1992).

Monoclonal antibody Z3 stabilized the capsule of the reference strains of serotypes 1, 1/2 and 2 (Fig. 4). Less capsular material was stabilized on type 1, as previously shown by Jacques et al. (1990) with polyclonal antibodies, compared to the other two types. This confirms results obtained in the ELISA-inhibition test.

**Involvement of mAb Z3 in opsonization, protection and clearance of S. suis serotype 2**

The phagocytic assay carried out in this study showed the opsonizing potential of mAb Z3. Results obtained with non-opsonized cells (31% ± 1%; mean ± sd, n = 3) were similar to those previously obtained in other studies (Charland et al., 1996). Interestingly, cells opsonized with mAb Z3 (48% ± 4% polyclonal serum (42% ± 3%) were significantly more phagocytosed by porcine blood monocytes (P < 0.005; analysed by Student's two-tailed unpaired t test). Increased amounts of mAb Z3 (800 μg) did not increase the phagocytosis rate (45% ± 2%; P > 0.05). Despite the fact that more bacteria were phagocytosed after opsonization with anti-capsular antibodies, the values obtained in this study were lower than expected. A clear correlation between encapsulation and resistance to phagocytosis has been demonstrated by us (Charland et al., 1997; Brazeau et al., 1996) and by other workers (Wibawan & Lämmler, 1994; Quessy et al., 1994b). However, there is no published report on phagocytosis obtained after opsonization with serotype-specific antibodies.

In the protection assay, 2 d post-inoculation, 15 out of 15 mice in the negative control group were seriously ill or...
Monoclonal antibody against *S. suis* serotype 2

**Fig. 4.** Transmission electron micrograph of thin sections of cells of *S. suis* serotypes 1 (a), 1/2 (b), 2 (c) and 3 (d; negative control) incubated with monoclonal antibody Z3 and stained with ruthenium red. Bar, 200 nm.
Table 1. Evaluation of the protective activity of a Streptococcus suis serotype 2 specific monoclonal antibody Z3, rabbit polyclonal anti-type 2 antibodies and isotype-matched, non-specific monoclonal antibody 5.1 G8 F10 using a standardized murine model of infection

<table>
<thead>
<tr>
<th>No. of bacteria injected (c.f.u. ml⁻¹)</th>
<th>Antibody</th>
<th>No. of affected mice/no. of inoculated mice</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁰</td>
<td>None</td>
<td>15/15 (<em>P</em> &gt;0.05)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Z3 (40 mg kg⁻¹)</td>
<td>13/15 (<em>P</em> &gt;0.05)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Polyclonal</td>
<td>0/15</td>
<td>100</td>
</tr>
<tr>
<td>10⁷</td>
<td>None</td>
<td>5/15 (<em>P</em> &lt;0.0005)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Z3 (40 mg kg⁻¹)</td>
<td>0/15 (<em>P</em> &lt;0.0005)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5.1 (40 mg kg⁻¹)</td>
<td>15/15</td>
<td>0</td>
</tr>
</tbody>
</table>

ND, Not determined.

dead as well as 13 out of 15 mice of the group injected with mAb Z3 (Table 1). Since antibody concentrations used in similar experiments by other workers (Ricci et al., 1996; Teti et al., 1992) did not show any protective activity in our hands, a higher amount of mAb was used to ensure that a failure in protection was not due to a low antibody concentration. All mice injected with the polyclonal serum survived and did not show any clinical signs. Inoculation of fewer bacteria showed that the mAb could, at least partially, protect mice (Table 1) whereas mice injected with mAb 5.1 G8 F10 (40 mg kg⁻¹) as a negative control were not protected. All mice injected with mAb Z3 survived and did not show clinical signs whereas those injected with the control mAb were seriously affected or died. These results indicate that protection induced by mAb Z3 was specific and not due to a high amount of IgG. Injection of other mice with 40 mg mAb Z3 kg⁻¹ mixed with bacterial suspensions of types 1 and 1/2 showed similar results (data not shown).

Fig. 5 shows that mAb Z3 had a possible role in elimination of bacteria in vivo. As soon as 2 h after injection, mAb Z3 helped completely to clear circulating bacteria, whereas in mice injected with S735 alone, a high number of bacteria could still be recovered 48 h after injection. Mice injected with mAb 5.1 G8 F10 as a negative control did not show any decrease in the number of circulating bacteria, unlike mAb Z3, indicating that clearance was antibody-specific. However, it cannot be ruled out that some differences in clearance of infection between the test and control groups might have been a reflection of micro-agglutinations of the bacteria, since mice were injected with a mixture of cells and mAb. Clearance may have been accelerated by removal of agglutinations of bacteria, in both protection and clearance assays.

Capsular material is the first bacterial component to be met by the host defence mechanisms. It is one of the first constituents to stimulate the immune system of an individual. However, in pigs infected with S. suis serotype 2, very few anti-capsular antibodies could be detected even though they are protected against a challenge with a virulent strain (Del Campo Sepulveda et al., 1996). Interestingly, Kebede et al. (1990) could not obtain any protection against serotypes 1 and 2 using a live 1/2 vaccine. Since a live vaccine was used in this study, it is possible that other factors such as common antigens (proteins) as well as a cellular immune response helped to provide protection. Elliott et al. (1980) could elicit opsonizing antibodies in pigs vaccinated with purified capsular polysaccharides although they did not test their protective activity whereas Holt et al. (1990b) could stimulate anti-capsule antibody production, but failed to stimulate immunity. Thus, although antibodies to capsular material are shown to be protective against many bacterial pathogens on their own (Hurpin et al., 1992; Ricci et al., 1996), the situation may be different for S. suis. Indeed, in addition to the capsule, other structures, such as bacterial proteins (Holt et al., 1990a;
Quessy et al., 1994a), are probably involved in the induction of a complete protection. However, bacterial doses injected in mice are, in general, quite high and it is conceivable that mAb Z3 could protect pigs against a natural challenge which may involve lower doses of bacteria.

Pathogenesis of S. suis infections differs from that of other streptococcal infections (Wessels et al., 1989; Williams & Blakemore, 1990). In fact, although S. suis possesses capsular sialic acid, like group B streptococci, sialic acid does not seem to be involved in resistance to phagocytosis as for group B streptococci (Wessels et al., 1989). Capsular sialic acid also plays a role in the pathogenesis of other meningitis-causing bacteria such as Escherichia coli K1 (Kim et al., 1992) and group B Neisseria meningitidis (Jarvis & Vedros, 1987). Interestingly, these two bacteria have immunochemically identical capsules (Kasper et al., 1973). The sialic acid concentration in S. suis strains seems to be considerably lower than in other bacterial species which might explain, at least in part, differences seen between results with mAb Z3 and results with mAb of group B streptococci (Egan et al., 1983; Teti et al., 1992).

Concluding remarks

In conclusion, a mAb directed to a S. suis capsular epitope, possibly the sialic acid moiety, was shown to have opsonizing capacities. In addition, this mAb helped to eliminate bacteria from circulation in mice. However, protection in a murine model could only be observed when mice were challenged with a sublethal dose of bacteria. Antibodies against other structures, such as cell-wall proteins, are probably needed to obtain complete protection against infection due to S. suis serotype 2.

ACKNOWLEDGEMENTS

We wish to thank M. Marcoux, D. Meunier-Côté and B. Foiry for their excellent technical assistance and Dr R. Higgins for reviewing the manuscript. This work was supported by grant OGP0154280 from the Natural Sciences and Engineering Research Council of Canada (NSERC) and by grant 97-NC-1037 from the Fonds pour la Formation de Chercheurs et l'Aide à la recherche. N.C. is the recipient of a NSERC scholarship.

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Received 22 April 1997; revised 24 July 1997; accepted 5 August 1997.