***In vitro* phagocytosis and survival of *Streptococcus suis* capsular type 2 inside murine macrophages**

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In this study, data on phagocytosis of *Streptococcus suis* and its survival inside macrophages are presented. Mouse peritoneal macrophages were incubated in the presence of one of five different strains of *S. suis* capsular type 2: a virulent wild-type strain (1591), a non-capsulated non-virulent mutant strain (M2), a poorly capsulated non-virulent mutant strain (M42), a non-virulent capsulated strain (1330), and the wild-type reference (virulent) strain S735. Opsonized or non-opsonized bacteria were incubated with macrophages *in vitro* and samples were obtained after 1 and 3 h incubation. Phagocytosis as well as live and dead intracellular organisms were determined by acridine orange and crystal violet staining. After 1 h incubation, non-opsonized virulent and non-virulent capsulated bacteria were poorly phagocytosed (by less than 7% of the macrophages), whereas the non-capsulated non-virulent mutant strain was highly phagocytosed (by more than 68% of the macrophages). The M42 mutant strain was more phagocytosed than the capsulated strains but less than the non-capsulated M2 mutant strain (35%). In contrast, a higher percentage of live bacteria was observed inside macrophages for the capsulated strains (1591 and S735) than for the non- or poorly capsulated mutant strains (M2 and M42). Opsonization of bacteria with rabbit serum or heat-inactivated rabbit serum significantly increased phagocytosis. For every opsonized strain, after 3 h incubation, the percentage of live bacteria within macrophages was considerably lower than the corresponding non-opsonized strains. In conclusion, the capsule of *S. suis* type 2 appears to act as an important anti-phagocytic factor. However, virulent capsulated non-opsonized strains can be phagocytosed by mouse peritoneal macrophages within which they appear to survive for at least 3 h. Serum factors other than complement increase not only phagocytosis but also intracellular killing of *S. suis* of both capsulated and non-capsulated strains.

Keywords: *Streptococcus suis*, capsule, opsonization, phagocytosis, intracellular survival

INTRODUCTION

Streptococcus suis is responsible for a wide range of infections in swine, such as meningitis, septicaemia, arthritis, pericarditis, endocarditis, polyserositis and pneumonia (Higgins *et al.*, 1992; Alexander, 1991). In addition, other animal species (Devriese *et al.*, 1990) as well as humans (Arends & Zanen, 1988) can be affected.

S. suis shares antigens with Lancefield group D streptococci but appears to be genetically different (Kilpper-Balz & Schleifer, 1987). So far, 35 capsular types have been described (Gottschalk *et al.*, 1989, 1991a; Higgins *et al.*, 1995); among them, the capsular type 2 is the most frequently isolated from diseased pigs and humans (Arends & Zanen, 1988; Higgins *et al.*, 1992).

Attempts to control the infection caused by *S. suis* capsular type 2 in the field by vaccination have given equivocal results, partially due to the lack of knowledge on bacterial virulence factors. Different bacterial structures, such as

Abbreviations: AO, acridine orange; CV, crystal violet; HBSS, Hanks' Balanced Salt Solution; TEM, transmission electron microscopy.

capsule, fimbriae, haemagglutinins, extracellular and cell wall-associated proteins and haemolysin have been suggested as virulence factors (Gottschalk *et al.*, 1990, 1992, 1995; Vecht *et al.*, 1991; Jacobs *et al.*, 1994). The capsule is recognized as an important component for successful invasion and disease production by many bacterial pathogens (Rubens *et al.*, 1987). The capsular polysaccharide of *S. suis* serotype 2 is composed of five sugars, including sialic acid, and has a molecular mass of 100 kDa (Elliott & Tai, 1978). Although capsular sialic acid plays a well-recognized role in the resistance to phagocytosis in group B streptococci, as demonstrated by the study of asialo mutants (Rubens *et al.*, 1987), similar functions in *S. suis* infections have not yet been studied. It has been reported that isolates of *S. suis* from diseased animals possess a thicker capsule than those isolated from healthy animals (Gottschalk *et al.*, 1991b). In addition, an increased thickness of the capsule and an increased resistance to killing by swine polymorphonuclear neutrophils was noted for virulent strains, but not for non-virulent strains, after being cultured *in vivo* (Quessy *et al.*, 1994). It is not known whether this resistance was due to a reduced phagocytosis rate.

In contrast, Williams (1990) reported that both virulent and non-virulent capsulated isolates were readily phagocytosed by murine macrophages, but only virulent strains survived within phagosomes. Since in this study he compared capsulated virulent and non-virulent strains, the role of capsulation in phagocytosis and intracellular survival of *S. suis* could not be examined. Consequently, it was proposed that *S. suis* enters into circulation from the tonsils, is taken up by monocytes and transported to the cerebrospinal fluid via the choroid plexus (Williams & Blakemore 1990a-c).

To study the role of the capsule of *S. suis* in pathogenicity we examined the phagocytosis and intracellular survival of capsulated and non-capsulated *S. suis* capsular type 2 strains by murine macrophages.

METHODS

Bacterial strains. Five strains of *S. suis* capsular type 2 were used. The reference strain S735 (NCTC 10234) was kindly provided by Dr Robert Higgins, University of Montreal, Canada. This strain is virulent and highly capsulated (Jacques *et al.*, 1990; Quessy *et al.*, 1995), and was first isolated from a diseased pig in The Netherlands. Wild-type strain 89-1591, which is also virulent and highly capsulated, was originally isolated from a case of septicaemia and meningitis in a pig. Strains M2 and M42 are spontaneous mutants derived from strain 1591 (Gottschalk *et al.*, 1992). Strain M2 is non-virulent and non-capsulated and strain M42 is non-virulent and partially capsulated. Finally, a capsulated non-virulent strain (1330) (Quessy *et al.*, 1995), which was isolated from the lungs of a diseased pig, was also included in this study. All isolates were cultured on 5% (v/v) bovine blood agar plates at 37 °C for 18 h. From this culture, four colonies were inoculated into 5 ml Todd-Hewitt broth (THB) (Difco Laboratories) and incubated at 37 °C for 18 h. Fifty microlitres of this broth was used to inoculate 5 ml fresh THB and culturing was by incubation at 37 °C for 5–6 h with agitation. The cells were harvested by centrifugation at 3000 g

for 10 min and resuspended in 5 ml RPMI 1640 (Gibco BRL) medium with glutamine and 25 mM HEPES, lacking sodium bicarbonate. The final concentration was adjusted to OD₅₄₀ 0.03 (Titertek Multiskan; Flow Laboratories), which corresponds to approximately 5×10^6 c.f.u. ml⁻¹.

For opsonization studies, inactivated or non-inactivated non-immune fresh rabbit serum (20%, v/v) was added to the bacterial suspension and the whole mixture was rotary shaken for 30 min at 37 °C. Inactivation of serum was carried out at 57 °C for 30 min.

Preparation of macrophages. Elicited murine macrophages were obtained using standard procedures (Adams *et al.*, 1981). Briefly, five or six, 6–8-week-old CF1 male mice (Charles Rivers) were used per individual assay. Mice were inoculated intraperitoneally with 1.5 ml 3% (w/v) thioglycolate sterile broth (Sigma). Macrophages were recovered 4 d later by washing the peritoneal cavity with 5.0 ml Hanks' Balanced Salt Solution (HBSS) Ca²⁺-, Mg²⁺-free; (Gibco). They were pooled, pelleted by centrifugation at 1000 g for 10 min and resuspended in 5.0 ml culture medium (RPMI 1640 with glutamine and 25 mM HEPES, lacking sodium bicarbonate) supplemented with 10% heat-inactivated foetal calf serum (Gibco) pre-warmed to 37 °C. The concentration was adjusted to 0.5×10^6 cells ml⁻¹ and 1.0 ml of this macrophage suspension was placed per well of a tissue culture plate of 24 wells containing 13 mm circular glass coverslips and incubated in a humid atmosphere for 45 min at 37 °C in 5% (v/v) CO₂. Non-adherent cells were removed by three washings with HBSS. To evaluate the proportion of the macrophages harvested, a non-specific esterase staining was performed according to the method of Koski *et al.* (1976).

Phagocytosis assay. Two isolates were tested in triplicate per individual assay, and each isolate was tested at least twice. In each assay, three coverslips were placed in wells, covered with 1.0 ml bacterial suspension to obtain a ratio of about 10 bacterial cells per macrophage, and incubated for 60 min in humid atmosphere at 37 °C, in 5% (v/v) CO₂. Coverslips were washed five times with HBSS to remove the extracellular non-adherent bacteria. To evaluate the percentage of phagocytosis and the phagocytosis capacity (see below), some coverslips were stained after 1 h incubation. The three coverslips were stained with acridine orange (AO; Sigma) (0.144 mg ml⁻¹ in HBSS, pH 7.2) for 45 s, washed twice, stained with crystal violet (CV; Sigma) (0.50 mg ml⁻¹ in 0.15 M NaCl, pH 7.2) for 45 s, washed twice and rinsed with a 3% (w/v) gelatin solution. To evaluate intracellular survival, after the 60 min incubation, extracellular bacteria were killed with 0.45 µg penicillin G ml⁻¹ which corresponds to 15-times the MIC for the strains tested in this study (Hand & King-Thompson, 1986). The coverslips were then incubated for a further 2 h.

Coverslips were observed under oil at a magnification of $\times 1000$ using a Leitz microscope with a mercury lamp source with a 450–490 nm excitation filter. The proportion of cells containing ingested bacteria (percentage of phagocytosis) was recorded from 200 macrophages after 1 h incubation. The number of phagocytosed bacteria per macrophage (phagocytosis capacity) was recorded in 30 macrophages containing ingested bacteria after 1 and 3 h incubation. Distinction was made between live (green fluorescence) and dead bacteria (red fluorescence). To evaluate the intracellular survival, an AO–CV stain was used. Vital staining with AO is a very sensitive method for detecting ingested bacteria and also allows differentiation between live and dead intracellular bacteria since dead bacteria fluoresce red (metachromasy) and live bacteria fluoresce green or light yellow (orthochromasy). CV staining quenches the fluorescence of

extracellular bacteria, while the fluorescence of intracellular bacteria remains unaffected. Observation and quantification of live and dead bacteria were always done by the same person. Green or light yellow bacteria were considered as live bacteria, while red, orange and orange-yellow bacteria were recorded as being dead. The results were displayed as a ratio of the total number of live bacteria to the total number of dead bacteria.

Transmission electron microscopy (TEM). For TEM studies, plastic coverslips were used instead of glass coverslips. At the end of the phagocytosis assay, the cells adherent to the coverslips were fixed with 2.5% (v/v) glutaraldehyde in HBSS buffer, pH 7.0 for 1 h, and post-fixed with 1% (w/v) osmium tetroxide for 45 min. Dehydration was performed in a graded ethanol series and the coverslips were embedded in Spurr. Silver interference-colour sections were collected over copper grids coated with Formvar, and post-stained with silver nitrate and lead citrate. Sections were examined and photographed with a Philips 101 transmission electron microscope operating at 80 kV.

Statistical analysis. The Fischer test was performed for the percentage of phagocytosis and the phagocytic capacity. For survival of bacteria inside macrophages the Wilk's Lambda test was performed. This test is a multivariate analysis of variance to simultaneously test two series of data (the numbers of live and dead bacteria).

RESULTS

Phagocytosis of *S. suis*

The percentage of macrophages in the harvesting of peritoneal cells was 97%. Elicited mouse peritoneal macrophages cultured in the presence of different strains of *S. suis* capsular type 2 for 1 h were able to phagocytose the bacteria (Table 1). The number of macrophages which contained ingested bacteria varied according to the strain and to opsonization of the bacteria. In the absence of opsonization, the number of macrophages which ingested capsulated *S. suis* (1330, 1591, S735) was significantly lower ($P < 0.0001$) than that containing the non-capsulated (M2) and the poorly capsulated (M42) strains

(Table 1). Only a small number of macrophages was able to ingest the virulent and non-virulent highly capsulated strain. Indeed, 5.5% of the macrophages ingested the capsulated non-virulent (1330) strain, while 7% of the macrophages ingested each of the capsulated virulent strains (1591, S735). The number of macrophages phagocytosing the partially capsulated strain (M42) was higher than the capsulated strains, but lower than the non-capsulated strains ($P < 0.0001$). After 3 h incubation, the percentage of macrophages containing intracellular *S. suis* was similar to that after 1 h incubation.

Opsonization increased the phagocytosis of all the strains (Table 1). As was the case for non-opsonized bacteria, no difference was observed between capsulated virulent and capsulated non-virulent strains. From the Fischer test used to analyse the percentage of phagocytosis and the phagocytic capacity, it appeared that all the strains behaved in a similar way according to opsonization with fresh serum or with heat-inactivated serum. In other words, there was an increase of the percentage of phagocytosis and phagocytic capacity with opsonization (with fresh and heat-inactivated serum), but this increase was similar for every strain ($P > 0.01$). The less capsulated and the non-capsulated strains were phagocytosed by higher numbers of macrophages. Elimination of the complement in the serum by heating did not affect the phagocytosis of any strains as compared with the phagocytosis of strains opsonized with fresh serum.

Phagocytic capacity

Since the numbers of bacteria (strains 1330, 1591, S735) present inside individual macrophages was low, the number of ingested bacteria was recorded per 30 macrophages which showed ingested bacteria after 1 and 3 h incubation (Figs 1 and 2). For both incubation delays, the phagocytic capacity was significantly higher for the non-capsulated and partially capsulated mutants than for the capsulated virulent and capsulated non-virulent strains ($P < 0.0001$), and this independently of the opsonization treatment. No significant difference ($P < 0.0001$) was observed between the capsulated virulent and capsulated non-virulent strains. Although the numbers of ingested M2 isolates seem to be greater after 3 h than after 1 h incubation, these differences were not significant (they were due to some counting difficulties of the intracellular bacteria resulting in high standard deviations). Indeed, especially for M2 isolates, the numbers of intracellular bacteria were very high (more than 30 bacteria per macrophage) and they tended to form clusters within the cytoplasm rendering differentiation of individual bacteria difficult.

The ratios of live to dead bacteria per 30 macrophages with ingested micro-organisms after 1 and 3 h incubation were compared (Table 2). These numbers were different according to the virulence and according to opsonization. In the absence of any opsonization, the ratio of live to dead bacteria for virulent strains which were ingested in macrophages after 1 h incubation was increased after an additional 2 h incubation period ($P < 0.0001$; calculated

Table 1. Phagocytosis of *S. suis* serotype 2 by mouse peritoneal macrophages after 1 h

Strain*	No. of macrophages which phagocytose bacteria†		
	Non-opsonized	Opsonized with non-inactivated serum	Opsonized with heat-inactivated serum
1591	14 ± 4‡	34 ± 5	24 ± 11
S735	14 ± 7	35 ± 12	29 ± 7
1330	11 ± 3	29 ± 2	23 ± 4
M2	136 ± 12	167 ± 21	171 ± 25
M42	71 ± 23	105 ± 3	105 ± 9

*1591 and S735, virulent capsulated strains; 1330, non-virulent capsulated strain; M2, non-virulent non-capsulated mutant of 1591; M42, non-virulent partially capsulated mutant of 1591.

† A total of 200 macrophages was observed.

‡ Mean of at least three separate experiments ± SD.

Fig. 1. Number of *S. suis* capsular type 2 phagocytosed within 30 macrophages after 1 h incubation. Details of strains are given in the footnotes to Table 1. Data are means of three to six experiments; vertical bars represent standard deviation. □, Non-opsonized; ■, opsonized with non-inactivated serum; ▨, opsonized with heat-inactivated serum.

Fig. 2. Number of *S. suis* capsular type 2 phagocytosed within 30 macrophages after 3 h incubation. Details of strains are given in the footnotes to Table 1. Data are means of three to six experiments; vertical bars represent standard deviation. □, Non-opsonized; ■, opsonized with non-inactivated serum; ▨, opsonized with heat-inactivated serum.

with unrefined data). For the non-virulent strains, the situation was different, the ratio of live to dead bacteria inside macrophages decreased but to a lesser extent for the capsulated non-virulent strain (1330) than the non- or poorly capsulated mutants strains (M2, M42).

After opsonization with fresh or heat-inactivated serum the ratio of live to dead bacteria within the macrophages decreased with the prolongation of the incubation period for all the strains (Table 2). Thus opsonization diminishes intracellular survival for all the strains. However, the ratio for strain S735 was considerably higher than for the other capsulated strains after 1 h incubation.

Location of *S. suis* within the macrophages

Observation of thin sections by TEM revealed that *S. suis* was located within membrane-bound vesicles in the macrophage cytoplasm (Fig. 3). Since these vesicles had a clear content, they were considered as being phagosome-like vesicles. No morphological differences could be observed between virulent micro-organisms phagocytosed for 1 or 3 h. Replication of virulent micro-organisms was observed after 1 or 3 h incubation (Fig.

3b). Strains M2 and M42 demonstrated evident degradation of the cell wall after 3 h incubation (Fig. 3d). The membrane of the phagosomes containing non-virulent M2, M42 and 1330 strains was closely apposed to the micro-organisms (Fig. 3c, d), while a space could be observed between the phagosome membrane and the virulent strain S735 (Fig. 3a, b).

DISCUSSION

Capsule is recognized as an important component for successful host invasion and disease production by bacterial pathogens (Rubens *et al.*, 1987). One of the best known mechanisms by which capsules protect bacteria is impairing their interaction with phagocytic cells. Since *S. suis* possesses a well-developed polysaccharidic capsule which, in addition, is rich in sialic acid (an antiphagocytic factor) (Elliott & Tao, 1978), a natural resistance to phagocytosis, in the absence of specific antibodies, would be logical. However, Williams (1990) reported in an *in vitro* study, that well-capsulated non-pathogenic and pathogenic strains of *S. suis* were phagocytosed equally by macrophages in the absence of antibodies and/or comp-

Table 2. Persistence of live *S. suis* serotype 2 within macrophages after 1 and 3 h incubation

Strain*	Ratio of live/dead bacteria†					
	Non-opsonized		Opsonized with fresh serum		Opsonized with heat-inactivated serum	
	1 h	3 h	1 h	3 h	1 h	3 h
1330	0.31	0.16	0.30	0.10	0.36	0.16
1591	0.41	0.53	0.41	0.15	0.38	0.14
S735	0.57	0.96	2.99	0.60	1.83	0.64
M2	0.14	0.05	0.56	0.08	0.31	0.06
M42	0.12	0.03	0.08	0.03	0.13	0.06

* See footnotes to Table 1 for strain characteristics.

† In 30 macrophages which have phagocytosed bacteria.

lement. The phagocytosed non-pathogenic strains did not survive whereas phagocytosed, pathogenic strains survived and could even grow intracellularly. From these observations and from *in vivo* studies in pigs, Williams & Blakemore (1990a-c) postulated that, although both polymorphonuclear and macrophage cells are capable of phagocytosing pathogenic *S. suis* in pigs lacking anti-*S. suis* antibodies, only pathogenic bacteria could survive and multiply inside macrophages.

The present study confirms the results presented by Williams (1990), since well-capsulated bacteria were phagocytosed by less than 10% of macrophages in the absence of antibody and/or complement in similar rates independently of their virulence capacity. However, the non-capsulated and the poorly capsulated strains were significantly more phagocytosed than the well-capsulated strain. This suggests that the capsule plays an anti-phagocytic role in *S. suis* and confirms the observation of Williams (1989) that a partially capsulated mutant is more phagocytosed by macrophages than capsulated isolates. Since in our experiment the non-capsulated and poorly capsulated strains were spontaneous mutants, other unknown factors could eventually also play a role. Similar observations were recently made by Wibawan & Lammler (1994) who indicated that a non-capsulated strain of *S. suis* capsular type 1, either opsonized or non-opsonized, was phagocytosed to a greater degree than the capsulated strain.

Although the presence of complement improves phagocytosis of many bacteria (Horstman, 1992), in the present study complement did not have a significant influence on phagocytosis of *S. suis*. However, serum factors other than complement increased the ingestion and decreased the intracellular survival of *S. suis* within macrophages. Wibawan & Lammler (1994) had also observed an increase in phagocytosis when capsulated or non-capsulated strains were opsonized before phagocytosis.

Interestingly, there are some reports indicating that complement does not seem to play an important role in phagocytosis of group B and A streptococci (Shigeoka *et al.*, 1983; Wagner *et al.*, 1986). Indeed, S. Quessy and co-workers (personal communication) have identified and partially characterized an albumin-binding protein of *S. suis*. They also observed an increase in phagocytosis rate after treatment of *S. suis* with albumin. According to Wagner *et al.* (1986), plasma-protein binding may be involved in the pathogenesis of group A streptococcal infections. Interestingly, the virulence of *S. suis* capsular type 2 strains increased significantly when the bacterial inoculum was grown in 10% unactivated bovine serum (Beaudoin *et al.*, 1992).

Williams (1990) observed that non-pathogenic capsulated isolates were killed whereas intracellular capsulated pathogenic organisms survived and replicated within phagosomes in the absence of anti-*S. suis* type 2 antibody and complement. In this study, we partially confirmed these results since virulent, but not non-virulent non-opsonized strains could survive inside macrophages. When normal serum, inactivated or non-inactivated, was used for opsonization, neither virulent nor non-virulent strains were able to survive inside macrophages. These results differ from those of Williams (1990), who indicated that the hyperimmune rabbit serum, but not normal serum, against *S. suis* capsular type 2 increased intracellular killing of bacteria. However, in that study, sera were added after the phagocytosis took place.

The ratio of live to dead organisms was lower in our study than that reported by Williams (1990). Using Diff-Quick staining, we observed the remaining extracellular bacteria which could not be eliminated by washings (data not shown). Ingestion of these extracellular bacteria could probably influence these ratios. To avoid this effect, we added a high concentration of penicillin G, to which the strains of *S. suis* used in this study were highly susceptible. It has been previously demonstrated that this antibiotic does not have any activity inside macrophages during the 3 h incubation period (Hand & King-Thompson, 1986; Renard *et al.*, 1987). Under these conditions, we observed no visible extracellular bacteria (data not shown).

Several intracellular facultative or obligatory parasites are able to survive inside macrophages or monocytes, using different strategies (Goren, 1977; Horwitz, 1988; Kaufmann & Reddehase, 1989). The mechanism by which virulent capsulated strains of *S. suis* type 2 survive for 3 h within phagosomes remains unknown. This intracellular survival does not seem to be due to superoxide dismutase synthesis (Langford *et al.*, 1991).

Circulating monocytes containing bacteria-size particles migrate into the cerebro-spinal fluid compartment (Williams & Blakemore, 1990c). It has been shown that after intravenous inoculation of pigs with pathogenic *S. suis* type 2, circulating monocytes contain intracellular bacteria. In fact, it is generally accepted that in the pathogenesis of *S. suis* meningitis bacteria enter into the cerebrospinal fluid compartment in association with monocytes. However, Williams & Blakemore (1990c)

Fig. 3. Thin sections of murine macrophages containing *S. suis* capsular type 2. (a) and (b) Virulent capsulated strain after 1 h incubation; (c) non-virulent partially capsulated strain M42 after 3 h incubation; (d) non-virulent non-capsulated strain M2 after 3 h incubation. Arrows indicate the phagosome membrane. (a), (c) and (d) Bars = 1 μ m; (b) bar = 0.5 μ m.

showed that less than 2% of the monocytes of the bacteraemic pigs contained intracellular bacterial profiles. Finally, it is important to note that, in the present study with a ratio of 10 bacteria/1 macrophage, more than 80% of the macrophages could not phagocytose well-opsonized capsulated *S. suis*. This may indicate that in natural infection most bacteria remain outside macrophages or monocytes. Whether these bacteria are phagocytosed and destroyed by other host cells or whether they play an active role in the pathogenesis of the infection is not known.

In conclusion, the capsule of *S. suis* type 2 acts as an important anti-phagocytic factor. However, virulent

capsulated non-opsonized strains can be phagocytosed by mouse peritoneal macrophages within which they survive for at least 3 h. Serum factors other than complement increase not only phagocytosis but also intracellular killing of *S. suis*.

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