THE GROWTH AND SURVIVAL OF CAPSULATE AND NON-CAPSULATE BACTEROIDES FRAGILIS IN VIVO AND IN VITRO

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SUMMARY. The growth of capsulate and non-capsulate Bacteroides fragilis in chambers implanted in the mouse peritoneal cavity was compared. Capsulate and essentially non-capsulate ( < 1% capsulate) populations of B. fragilis strains NCTC9343 and NCTC10584 consistently grew exponentially to > 10⁹ cfu/ml within 24 h in vivo, and low numbers of capsulate bacteria were maintained in the essentially non-capsulate population; however, the degree of capsulation of the capsulate population decreased by more than 60%. B. fragilis ATCC23745 differed from strains NCTC9343 and NCTC10584 in that growth was unpredictable and only occurred in some of the implanted chambers. Capsule production by cells of strain ATCC23745 varied from chamber to chamber: sometimes the proportion of capsulate cells increased after prolonged implantation. This could occur with either an increase or decrease in viable numbers in vivo and also after in-vitro incubation of this strain in chambers. The survival of capsulate and non-capsulate B. fragilis strains NCTC9343 and ATCC23745 was compared in aerobic and anaerobic conditions in vitro. In anaerobic conditions, capsulate and non-capsulate strain NCTC9343 survived equally well, whereas capsulate ATCC23745 survived better than its non-capsulate variants. Capsulate populations of both strains survived better than non-capsulate in aerobic conditions.

INTRODUCTION

In-vitro studies indicate that the capsule of Bacteroides fragilis impairs phagocytosis; however, its role in resistance to serum killing is less clear (Reid and Patrick, 1984). Smith (1980) stressed the importance of relating in-vitro investigations to growth in vivo. Animal models of intra-abdominal bacteroides infections have been reported by several authors. A study with rats indicated that B. fragilis ATCC23745 induced intra-abdominal abscess formation to a greater extent than other Bacteroides spp. (e.g., B. distasonis). This was attributed to the polysaccharide capsule of B. fragilis, because extracted polysaccharide alone caused abscesses. However, the

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inocula included barium sulphate and sterilised rat caecal contents, and the bacteria 
were suspended in glucose broth with peptone and yeast extract (PYG) (Onderdonk et al., 1977). Kasper et al. (1980) used this model to obtain in-vivo passaged *B. fragilis* ATCC23745. O'Keefe et al. (1978) implanted punctured table-tennis balls into the rabbit peritoneal cavity. Three weeks later, a suspension of *B. fragilis* ATCC23745 in chopped-meat-glucose broth was inoculated into the table-tennis balls to provide a model for studies of the penetration and activity of penicillin G. McConville et al. (1981) challenged mice intraperitoneally with suspensions of sterile mouse faeces and a clinical isolate of *B. fragilis*. Sterile mouse faeces alone induced abscess formation, but more abscesses were produced when viable or heat-killed bacteria were added. PYG and sterilised mouse caecal contents are highly chemotactic for neutrophils in the absence of serum (Joiner et al., 1980).

In this paper we report the growth of capsulate and essentially non-capsulate populations of *B. fragilis in vivo* and compare the survival of capsulate and non-capsulate bacteria *in vitro*. As it is difficult to determine the importance of the additives included in some animal models, we used a modification of the method of Day et al. (1980), in which bacteria are suspended in a salt solution and enclosed in a membrane-filter bound chamber. This initially allows free passage of diffusible host and bacterial factors, but excludes phagocytes.

**Materials and methods**

**Bacterial strains.** *B. fragilis* NCTC9343 was supplied by the Department of Bacteriology, University of Edinburgh Medical School, and *B. fragilis* ATCC23745 by the American Type Culture Collection, Rockville, MD. *B. fragilis* NCTC10584 was a departmental stock culture. Bacteria grown from these original cultures are referred to as normal (N) populations. N populations of strains NCTC9343 and NCTC10584 are <1% capsulate, whereas strain ATCC23745 is c. 17% capsulate (Patrick and Reid, 1983). Bacteria recovered from the 0–20% interface of a Percoll gradient are referred to as capsule (C) and from the 60–80% interface as non-capsulate (NC).

**Animals.** Female C57 mice aged 8–12 weeks (given free access to food and water) were used in all experiments.

**Bacterial culture methods.** Cultures were incubated at 37°C in an atmosphere of 90% H₂ and 10% CO₂ in anaerobic jars. The standard anaerobic procedures of Collee et al. (1972) were used.

Stock cultures, grown in defined broth (Van Tassel and Wilkins, 1978), were snap-frozen in liquid N₂ and 1-ml portions were stored at −70°C or in liquid N₂. Capsulate bacteria, recovered from the 0–20% interface of a Percoll discontinuous density gradient (Patrick and Reid, 1983) and grown to late log-phase in defined broth, provided a C stock. This was stored as above.

Total viable counts were determined by seeding lysed human blood agar (LHBA) with six 20-µl drops from a standard 20 G 2-in steel cannula (Astell, London).

**Preparation of inocula.** Bacteria were grown to late log-phase in the defined broth. C cultures were then layered on to a cushion of 20% Percoll (Pharmacia, Fine Chemicals Inc., London), centrifuged at 2600 g for 20 min in a bench centrifuge, and the 100% C (supernatant) suspension removed. Non-capsulate (NC) suspensions were obtained from the 60–80% interface of a Percoll discontinuous density gradient.

Standard suspensions containing (2–5) × 10⁷ cfu/ml were prepared in quarter-strength Ringer solution (Oxoid) with 0·05% free base of cysteine (RS + cys) and checked with a Pye Unicam SP 800 spectrophotometer.

**In-vitro growth.** Triplicate tubes of defined broth were inoculated for each sampling time at a concentration of (2–5) × 10⁷ cfu/ml. Each set of replicate tubes was incubated in a separate anaerobic jar and only sampled once.
In-vivo growth. Day et al. (1980) described a model for in-vivo growth of bacteria in chambers implanted intraperitoneally in mice. We used this method when chambers containing strain ATCC23745 were prepared in an anaerobic cabinet (Forma Scientific Inc.; gas mixture 85% N₂, 10% H₂, 5% CO₂). Because the method leaves a gas space inside the chamber, the following modification was used in all other experiments when chambers were prepared in aerobic conditions.

Chambers were made from 10-mm lengths of 1-ml polypropylene syringe barrels (Becton and Dickinson, Dublin). Two holes were made in the sides of the chambers, towards each end, with a red-hot 25 G needle. A membrane filter (0.45-μm pore; Millipore, Harrow, Middlesex) was stuck aseptically on to each end of the barrel with UHU glue (Beecham UHU, Brentford, Middlesex) and the glue allowed to harden. The chambers were filled through the sides with standard bacterial suspensions. A 2-ml syringe with a 26 G needle was inserted into one of the holes and the chamber filled until all the air was excluded (final volume c. 0.2 ml). The two holes were then sealed with UHU glue and the chambers transferred into bijou bottles containing c. 4.5 ml of RS + cys. These were then stored in an anaerobic jar with an activated Gas Generating Kit (Oxoid) at room temperature until implanted into the peritoneal cavity of mice; (two chambers were implanted in each mouse). LHBA plates were spread with 1 ml of the RS + cys from the bijou bottles to check for leaking chambers.

Duplicate mice were killed after the required incubation period and the chambers removed. The membrane filters were punctured with a syringe needle and the chambers washed four times with 0.2 ml RS. The numbers of viable bacteria in each chamber were determined.

The degree of bacterial capsulation was determined directly by examination of the suspensions obtained from the chambers, and indirectly by examination of the late log-phase growth after one subculture in defined broth. Standard suspensions of <1% capsulate (N) B. fragilis strains NCTC9343 and NCTC10584, non-capsulate B. fragilis ATCC 23745 and capsulate populations of all three strains were used as inocula.

Bacterial survival in vitro. Five-ml volumes of standard suspensions in RS + cys of C and NC variants of B. fragilis NCTC9343 and ATCC23745 were incubated in bijou bottles aerobically and anaerobically at 37°C for 24 h. The initial and final viable counts were then compared. Survival of strain ATCC23745 in anaerobically-prepared chambers placed in bijou bottles containing RS + cys was also determined. Degree of capsulation was determined in a late log-phase subculture in defined broth.

Microscopy. Capsulation was determined by light microscopy with eosin-carbol fuchsin negative staining (Cruickshank, 1965) and by electronmicroscopy with ruthenium red staining (Springer and Roth, 1973).

The presence of phagocytes on the chamber membrane filters was determined by light microscopy of methanol-fixed filters stained with M.D.Diff-Quick solutions 1 and 2 (Merz and Dade AG, Switzerland).

Statistical analyses. Growth curves of capsulate and normal populations were compared by a two-way analysis of variance with linear regression. Growth rates were compared by a test of significance between the regressions and the length of the lag-phase by comparison of the blocks of data (F tests) with a Texas T1 59 programmable calculator (Parker, 1979). The survival of capsulate and non-capsulate bacteria in vitro was compared by Student's 't' test. The bars included in the figures represent the standard errors.

Results

Microscopy of bacteria grown in chambers in-vivo

Suspensions of bacteria obtained from the chambers were examined directly with eosin-carbol fuchsin staining. Bacteria grown in vivo were smaller than those grown in defined broth: for example, cells of strain NCTC9343 grown in vivo were c. 0.7 x 1.0-3.6 μm whereas cells grown in defined broth were 0.9 x 1.4-5.1 μm. It was, therefore, difficult to determine the proportion of capsulate cells accurately because the
Fig. 1.—Survival, growth and percentage capsulation of initially c. 17% capsulate B. fragilis ATCC23745 in vivo and in vitro in chambers constructed in anaerobic conditions: increase or decrease in total viable count (log_{10} CFU/ml) in vivo (I) and in vitro (i); percentage capsulation determined directly on in-vivo grown bacteria (O) and, after one in-vitro subculture of bacteria incubated in vivo (●) and in vitro (■). Each symbol represents the results from one chamber. ± = standard error in all figs.

eosin-carbol fuchsin stain sometimes shrinks to leave a bright halo. Bacteria grown in vivo were subcultured once in defined broth and the proportions of capsulate cells were compared with direct determinations on chamber suspensions.

Strains NCTC9343 and NCTC10584 grown in vivo showed an apparent decrease in the proportion of capsulate cells after only one subculture in vitro; this was quite reproducible in replicate experiments. However, the percentage capsulation of B. fragilis ATCC23745 grown in vivo increased to a varying extent on in-vitro subculture (fig. 1). Some of these bacteria may not produce capsules in vivo, while retaining the potential for capsule production, or the capsules may be too small to be seen with the light microscope. Ruthenium red staining and electronmicroscopy showed the presence of a thick dark-staining layer outside the membrane on some of the cells. This resembled the ruthenium red-staining capsule described by Kasper (1976).
Because of these anomalies, the proportion of cells grown in vivo capable of producing capsules after one in-vitro subculture was used as an indicator of capsulation for all three strains.

*Observation of the implanted chambers*

Filters from the ends of the chambers were stained by the Diff-Quick method and examined microscopically. There was a gradual build up of leukocytes on the outer
surfaces of the filters on chambers containing C and N populations. Leukocytes were not observed on the inner surfaces of the filters. After prolonged incubation, the chambers in which growth occurred became encased in fibrin and granulation tissue, as described by Day et al. (1980) in their work with chambers containing Staphylococcus aureus.

**Growth and survival of B. fragilis**

During incubation *in vivo* for 24 h, C and N populations of *B. fragilis* NCTC9343 grew exponentially to > 10⁹ cfu/ml and maintained high viable numbers for at least 22 days (fig. 2). Throughout this period the proportion of capsulate cells in the N population remained low. However, the proportion of capsulate cells in the C population steadily decreased during 22 days to that of the N population. In contrast,
C populations subcultured in defined broth maintained a high level of capsulation (Patrick and Reid, 1983). The C population had a significantly longer lag-phase than the N population in vivo, although the growth rates did not differ significantly. Growth in vivo was slower than in defined broth, with generation times for C bacteria of 2.06 h in vivo and 1.60 h in defined broth, and for N bacteria of 1.96 h in vivo and 1.42 h in defined broth. The difference in the lag-phase and the selection against capsulate cells in vivo was not attributable to poor survival in RS + cys. When incubated in vitro at 37°C for 24 h, C and N populations of B. fragilis NCTC9343 maintained similar viable numbers anaerobically, whereas the capsulate population survived better than the non-capsulate in aerobic conditions. B. fragilis NCTC10584 behaved similarly, but strain ATCC23745 did not (fig. 3).

B. fragilis ATCC23745 did not grow exponentially during incubation for 24 h in vivo (fig. 3), even if the chambers were constructed and filled in an anaerobic cabinet (fig. 1). Growth did occur in some chambers after prolonged incubation, but it was erratic. In-vivo incubation tended to increase the proportion of cells capable of producing capsules, whether growth occurred or not, but again this was inconsistent. The percentage capsulation also increased in some control chambers incubated in vitro.

Fig. 4.—Survival of 100% capsulate (C) and non-capsulate (NC) B. fragilis strains NCTC9343 and ATCC23745 incubated aerobically (Aero) and anaerobically (Anaero) in Ringer solution + free base of cysteine (0.05%) at 37°C for 24 h. Each bar represents the combined results from two separate experiments. ★ = difference between C and NC statistically significant (p = 0.05).
in RS + cys at 37°C (fig. 1). The results summarised in fig. 4 show that the capsule of ATCC23745 enhances survival aerobically and anaerobically; this could be relevant to the selection of a capsulate population.

DISCUSSION

In this model, *B. fragilis* grew in chambers implanted intraperitoneally in mice in the absence of phagocytes and without the addition of adjuvants such as barium sulphate, sterile mouse faeces or growth medium. However, the physical presence of the chamber may enhance bacterial growth as the presence of a foreign body in the form of a subcutaneously implanted polytetrafluoroethylene tube in guinea pigs enhanced the growth of *S. aureus* (Zimmerli et al., 1982).

The present study shows that, in the absence of phagocytes, non-capsulate *B. fragilis* strains NCTC9343 and NCTC10584 can proliferate *in vivo* and have a selective advantage over capsulate organisms. The reasons for this are not known, but do not relate to survival aerobically or anaerobically *in vitro* in Ringer solution with cysteine. Any differences in the growth rate of the capsule and non-capsulate variants will influence the proportions of these cell types in the population, as would the frequency of switching from one state to the other. The relative importance of these two factors remains to be determined. Rapid phase or form variation in surface structures of pathogenic bacteria, without loss of the potential for production, could be an advantage in a changing host environment (Mäkelä et al., 1980). In studies of *B. thetaiotaomicron* by Burt et al. (1978), capsule cells changed to non-capsulate at a frequency of $1.1 \times 10^{-2}$ and non-capsulate to capsulate at $1.4 \times 10^{-2}$ *in vitro* at 37°C. Capsulation is related to colony form in *B. thetaiotaomicron*, but no change in colony morphology of capsulate and non-capsulate *B. fragilis* has been observed in the present study.

*B. fragilis* ATCC23745 differed from the other two strains in that growth *in vivo* in implanted chambers was unpredictable. There was also a trend towards a higher proportion of capsulate cells with longer incubation, whether in-vivo growth occurred or not. O'Keefe et al. (1978) also reported a wide variation in viable counts of *B. fragilis* ATCC23745 *in vivo*. Table-tennis balls implanted intraperitoneally (IP) into rabbits were inoculated with 1 ml of broth culture containing $10^9-10^{10}$ cfu/ml. After incubation for 4 or 5 days the same dose of penicillin G was given to all the rabbits, and viable counts then ranged from $10^3-10^{10}$ cfu/ml. This variation probably occurred during the 4–5-day incubation period and was not attributable to antibiotic treatment, because penicillin G was biologically inactive in heavily infected (> $10^9$ cfu/ml) balls. *B. fragilis* ATCC23745 isolated from experimental intraperitoneal abscesses in rats (Kasper et al., 1980) and subcutaneous abscesses in mice (Simon et al., 1982) produced more capsular material than the same strain serially subcultured only on blood agar. *B. fragilis* ATCC23745, isolated from experimental subcutaneous abscesses in mice, maintained higher viable numbers in the rabbit intraperitoneal table-tennis ball model of O'Keefe et al. (1978) than *B. fragilis* ATCC23745 which had been subcultured ten times on blood agar (Simon et al., 1982). The present study shows that the degree of capsulation of strain ATCC23745 can also be enhanced without incubation *in vivo* and that the capsule enhances *in-vitro* survival aerobically and anaerobically.

Although the reasons for the different behaviour of strains NCTC9343 and
NCTC10584, and ATCC23745 are not clear, the following facts should perhaps be given further consideration. Strain ATCC23745 was isolated from pleural fluid and was initially named Sphaerophorus intermediate (Bergan and Hovig, 1968), whereas strain NCTC9343 was isolated from an appendix abscess and NCTC10584 from pus. The polysaccharide capsules of strains ATCC23745 and NCTC9343 have been shown to be chemically and immunochemically different, although the lipopolysaccharide is similar (Kasper et al., 1983). In-vitro cultures of strain ATCC23745 have a higher proportion of capsulate cells than strains NCTC9343 and NCTC10584 (Patrick and Reid, 1983). Spherical bodies with multilayered walls, sometimes associated with bacteriophage, have been observed in B. fragilis strain ATCC23745 but not in other strains examined (Silver et al., 1975; Reid, 1981). Booth et al. (1979) reported a phage-carrier state in strains of B. fragilis that produced thick capsules. When these cultures were cured of phage the proportion of capsulate cells decreased. The activity of bacteriophage in strain ATCC23745 could be a factor involved in the differences observed in the present study, but direct evidence for this has yet to be obtained.

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