Capsulation of in vitro and in vivo Grown Bacteroides Species

By SHEILA PATRICK,* JOHN H. REID AND ALAN COFFEY
Department of Microbiology and Immunobiology, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK

(Received 28 June 1985; revised 21 October 1985)

By centrifugation on a four step Percoll density gradient cells of Bacteroides species could be separated according to the size of extracellular structure. The difference in size was visible by both light and electron microscopy. Two structures were observed on Bacteroides fragilis by electron microscopy, namely a fibrous network and an electron dense layer. An electron dense layer was visible on Bacteroides ovatus only when stained with ruthenium red. B. fragilis cells grown in the mouse peritoneal cavity did not produce a large fibrous network. An electron dense layer was observed on some cells in the presence of ruthenium red stain and cells possessing this layer were phagocytosed in vivo.

INTRODUCTION

The obligate anaerobe Bacteroides fragilis is the Gram-negative anaerobic organism most commonly isolated from clinical infections, e.g. bacteraemia, wound, intra-abdominal and urogenital infections (Lindberg et al., 1979). Capsules have been associated with the virulence of a number of bacteria (Robbins et al., 1980) and the extracellular layers observed on B. fragilis may fulfil a similar function (Hofstad, 1984). Capsulation of B. fragilis and other Bacteroides species is subject to variation both in capsule size and in the number of cells producing capsules within a population (Babb & Cummins, 1978; Burt et al., 1978; Lindberg et al., 1979; Brook et al., 1984). The factors influencing these variations have yet to be determined, although capsule size can vary with the growth medium (Patrick & Reid, 1983). Two extracellular structures have been observed by electron microscopy, a narrow electron dense layer close to the outer membrane (Kasper et al., 1977; Lindberg et al., 1979; Reid, 1983; Brook et al., 1984) and a more extensive fibrous network (Patrick & Reid, 1983; Reid, 1983; Lambe et al., 1984). It has been suggested that the difference in the appearance of these structures is related to the condensation of hydrated extracellular material which occurs during preparation for the electron microscope (Lambe et al., 1984). However, these authors did not define the populations examined with respect to either the proportion of cells with extracellular structures or variation in the size of extracellular structure examined by wet India ink stain and light microscopy.

We have previously reported the separation of B. fragilis cells with different sizes of extracellular structures (or capsules) as defined by light microscopy and wet India ink negative stain (Patrick & Reid, 1983). We now report on the ultrastructure of these separated populations after electron microscopy and examine cells with initially large and small extracellular structures after 24 h growth in the mouse peritoneal cavity.

METHODS

Bacterial culture. B. fragilis (NCTC 9343) was supplied by the Department of Bacteriology, University of Edinburgh Medical School. B. fragilis (ATCC 23745) by the American Type Culture Collection, Rockville, Md, USA, and B. ovatus (ATCC 8483) and B. fragilis (NCTC 10584) were departmental stock cultures. Bacteria were

Abbreviations: EDL, electron dense layer; EM, electron microscopy; LM, light microscopy; OM, outer membrane; RR, ruthenium red.
grown in defined broth (van Tassel & Wilkins, 1978) and incubated at 37 °C in an atmosphere of H₂ (90%) and CO₂ (10%) in anaerobic jars. The standard anaerobic procedures of Collee et al. (1972) were used.

Separation of bacteria. Bacteria were grown to late exponential phase in defined broth and layered onto a Percoll (Pharmacia) discontinuous density gradient, centrifuged at 2600 g for 20 min in a bench centrifuge and the cells from each interface of the gradient were removed as previously described (Patrick & Reid, 1983).

Microscopy. Capsulation was determined by light microscopy with eosin-carbol fuchsin negative staining (Cruickshank, 1965). Bacteria were fixed for electron microscopy by washing and resuspending in 0.1 M cacodylate buffer (pH 6.8) containing 2.5% (v/v) glutaraldehyde and incubated for 1 h at 4 °C in the dark. The bacteria were then washed in 0.1 M cacodylate buffer containing 1% (w/v) osmium tetroxide and incubated at room temperature for 3 h in the dark. Where cultures were stained with ruthenium red (Springer & Roth, 1973) this was included at both fixation stages at a concentration of 1 mg ml⁻¹. Bacteria were then washed before dehydration in a graded series of alcohols as follows: 50%, 75%, 95% ethanol, absolute ethanol, water free absolute ethanol and finally two washings in propylene oxide. Each step lasted 10–15 min. Preparations were then embedded in Spurr resin and ultrathin sections were examined with a Philips 301 transmission electron microscope.

In vivo culture of bacteria. Chambers were constructed and implanted in the mouse peritoneal cavity as previously described (Patrick et al., 1984). The chambers were constructed with either 0.45 μm pore membrane filters (Millipore), which excluded phagocytes, or 3 μm pore filters, which allowed their entry.

RESULTS

Light microscopy

Light microscopy (LM) of Bacteroides species stained negatively with wet India ink or eosin-carbol fuchsin showed a range of capsule size, the largest being equivalent to the diameter of the cell (Patrick & Reid, 1983). Cells with the largest size of capsule were easily distinguished by LM and the proportion of cells with this structure apparently varied with the species and strain.

We separated cells with different capsule sizes on a four step Percoll gradient. Cells with large capsules were found at the 0–20% (top) interface, cells with intermediate sizes of capsules at the 20–40% and 40–60% interfaces and cells with no capsules by LM at the 60–80% (bottom) interface.

The pattern of bands observed with populations grown from laboratory stock cultures (normal populations) showed that B. fragilis NCTC 9343 and NCTC 10584 each had less than 1% of the bacterial population with large capsules. B. fragilis ATCC 23745 had 17% of cells with large capsules, whereas B. ovatus ATCC 8483 had 80% of cells with large capsules. Cells with

Fig. 1. Four step (20, 40, 60 and 80%) Percoll density gradients after centrifugation with B. fragilis NCTC 9343. Tube 1, pattern of bands when laboratory stock culture was centrifuged. Tubes 2–5, separation, on the same gradients, of cells from first subculture from each interface: 2, 0–20% (top) interface; 3, 20–40% interface; 4, 40–60% interface; 5, 60–80% (bottom) interface.
Table 1. Incidence of fibrous network and electron dense layer in *B. fragilis* (NCTC 9343) and *B. ovatus* (ATCC 8483) after density gradient separation

Results were obtained by electron microscopy after separation on a discontinuous Percoll gradient (see Methods): + +, extensive fibrous network; +, presence of structure; –, absence of structure.

<table>
<thead>
<tr>
<th>Gradient position</th>
<th>0–20% Interface</th>
<th>20–40% Interface</th>
<th>40–60% Interface*</th>
<th>60% Interface*</th>
<th>60–80% Interface*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><em>B. fragilis</em></td>
<td><em>B. ovatus</em></td>
<td><em>B. fragilis</em></td>
<td><em>B. ovatus</em></td>
<td><em>B. fragilis</em></td>
</tr>
<tr>
<td>Fibrous network</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Electron dense layer</td>
<td>+</td>
<td>–</td>
<td>+/–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>
| *B. ovatus* cells remained in the 60% Percoll layer and did not accumulate at either the 40–60% or the 60–80% interface.

Intermediate capsules were present in all cultures except *B. fragilis* ATCC 23745. If cells from the different interfaces were each subcultured in defined medium, the populations could be enriched to some extent for the cells that predominated at that interface. The results for *B. fragilis* NCTC 9343 are illustrated in Fig. 1. The identity of bacterial suspensions taken from the Percoll interfaces of the enriched cultures was confirmed with the API 20A identification system and suspensions were streaked out for single colonies to confirm the purity of the cultures. Therefore the distribution of bacterial cells on the gradients could alter depending on the culture inoculum and there was a degree of stability in capsule size during the first subculture.

**Electron microscopy of cells grown in vitro**

The results are summarized in Table 1.

Cells of *B. fragilis* and *B. ovatus* with large capsules, as defined by LM (0–20% Percoll interface), showed an extensive fibrous network which had a coarser appearance when stained with ruthenium red (RR) (Fig. 2). *B. fragilis* cells also had an electron dense layer (EDL) adjacent to the outer membrane (OM) (Fig. 2b, d).

Cells with less capsular material by LM (20–40 and 40–60% Percoll interfaces) had either a marginal fibrous network or an EDL (Figs 3 and 4). In the case of *B. ovatus* an EDL was observed only when stained with RR (Fig. 3).

Cells which lacked capsular material by LM (60–80% Percoll interface) had no extracellular structures in the case of *B. ovatus*; however, an EDL was present in *B. fragilis* (Figs 5 and 6). Therefore the range of capsule sizes observed by LM was also observed by EM and *B. fragilis*, defined as noncapsulate by LM, had an extracellular EDL.

**EM of *B. fragilis* grown in vivo**

*B. fragilis* taken from the 0–20% (Fig. 2) and 60–80% (Fig. 6) Percoll interfaces was grown for 24 h in chambers implanted in the mouse peritoneal cavity (Patrick et al., 1984). The chambers were constructed either to exclude or to allow the entry of phagocytic cells. EM indicated that neither bacterial population produced large amounts of the fibrous network in vivo. Cells had either small amounts of fibrous network or a narrow EDL or no outer layer, both in the presence (Fig. 7a, b) and absence (Fig. 8a, b) of phagocytes. Where phagocytes entered the chamber, it was apparent that cells with and without the EDL were phagocytosed (Fig. 7a, b).

All the results shown are representative of at least three replicate experiments.
Fig. 2. Electron micrographs of cells from the 0–20% Percoll interface to illustrate the fibrous network (arrowed) on B. ovatus ATCC 8483 (a) and B. fragilis ATCC 23745 (b), and the electron dense layer (EDL) adjacent to the outer membrane (OM) of B. fragilis ATCC 23745, visible without ruthenium red (RR) stain. Note the coarser appearance of the fibrous network after RR staining on B. ovatus ATCC 8483 (c) and B. fragilis ATCC 23745 (d). Bar markers, 0.5 μm in micrographs and 100 nm in insets showing detail of the outer layers. Abbreviations are the same for all figures.
Capsulation of Bacteroides species

Fig. 3. Electron micrographs of B. ovatus ATCC 84833 from the 20-40% Percoll interface to illustrate the small amounts of fibrous network (arrowed) visible in the absence of RR stain (a) and the EDL and fibrous network observed after RR staining (b). Bar markers, see Fig. 2.
Fig. 4. Electron micrographs of *B. fragilis* NCTC 9343 from the 20-40% Percoll interface, not stained with RR (a) and from the 40-60% Percoll interface, stained with RR (b). Note that cells have either an EDL or a small fibrous network and that these are both visible in the absence of RR stain. Bar markers, see Fig. 2.
Capsulation of Bacteroides species

Fig. 5. Electron micrograph of *B. ovatus* ATCC 8483 from the 60% Percoll layer stained with RR. Note the absence of an EDL. Bar markers, see Fig. 2.

Fig. 6. Electron micrograph of *B. fragilis* NCTC 9343 from the 60–80% interface without RR stain. Note that an EDL is present on some cells. Bar markers, see Fig. 2.
Fig. 7. Electron micrographs of B. fragilis NCTC 10584 after incubation for 24 h in the mouse peritoneal cavity in the presence of phagocytes. Inoculum taken from the 0-20% Percoll interface. Note the absence of any extensive fibrinous network and the presence of an EDL on a cell apparently inside a phagocytic vacuole (b). Bar markers, see Fig. 2.
Capsulation of Bacteroides species

Fig. 8. Electron micrographs of RR stained B. fragilis NCTC 9343 after incubation for 24 h in the mouse peritoneal cavity in the absence of phagocytes. Inoculum taken from (a) the 0–20% Percoll interface and (b) the 60–80% Percoll interface. Note the absence of any extensive fibrous network. Bar markers, see Fig. 2.
Some of the structural differences in capsular material observed in the present study reflect different degrees of condensation with RR stain (Fig. 2); in the case of *B. ovatus* (Fig. 3) electron dense material was observed only with RR stain. Lambe *et al.* (1984) suggested that the fibrous network or glycocalyx condensed to form an EDL where it was not connected to more than one cell. However, an EDL and a marginal fibrous network were observed on adjacent cells of *B. fragilis* (Fig. 4), which by LM had similar sizes of capsules. Also, *B. fragilis* from the 60–80% Percoll interface lacked any capsule by LM, yet possessed an EDL by EM. Therefore it is possible that in some cases the EDL and fibrous network are different in nature. The ability of the cells to produce these different sized capsules is a relatively stable trait because subculture from the Percoll interfaces enriched populations for a particular size of capsule (Fig. 1).

An EDL of approximately 35 nm has been observed outside the OM in clinical isolates of *B. fragilis* and some strains of *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* by EM after RR staining (Kasper *et al.*, 1977, 1979). Similar structures have been reported by Lindberg *et al.* (1979) and Brook *et al.* (1984), but none of these authors reported the presence of fibrous material. Babb & Cummins (1977) observed capsules one to four times the diameter of the cell by negative stain and LM. These authors considered it likely that this structure differed from the RR stained layer described by Kasper *et al.* (1977). These anomalies could be due to differences in the composition of the culture media (Patrick & Reid, 1983). However, some of the pili-like structures observed by Brook *et al.* (1984) in thin sections could be coarse fibrous network similar to that observed after RR staining of cells from the 0–20% interface (Fig. 2c, d).

*B. fragilis* ATCC 23745, which possessed the RR staining layer, was reported to induce abscesses in a rat model of intra-abdominal infection, whereas *Bacteroides* species lacking this layer (e.g. *B. vulgatus*) did not. A crude preparation of polysaccharide material from strain ATCC 23745, which was thought to correspond to the RR staining layer, produced abscesses in the rat model in the absence of live bacteria (Onderdonk *et al.*, 1977). Subsequent investigations, however, indicated that this crude extract contained both capsular material and lipopolysaccharide (Kasper *et al.*, 1983). It was therefore unclear which of the outer layers of *B. fragilis* were the important virulence determinants. In the present study, an EDL was observed on some of the organisms grown *in vivo*. Large amounts of fibrous material were not produced in the mouse model of infection, although some of the cells retained the ability to do so. We have previously shown that after subculture in defined medium the fibrous network reappeared on up to 40% of the 0–20% interface cells grown *in vivo* but on less than 1% of the 60–80% interface cells (Patrick *et al.*, 1984). The lack of fibrous network *in vivo* may relate to the nutrients available in the intraperitoneally implanted chambers. It remains to be seen, however, why a population with the ability to produce a fibrous network is not selected, as studies *in vitro* indicate that the fibrous network confers resistance to phagocytosis and this resistance is related to the total amount of fibrous network present; preparations from the 60–80% interface were phagocytosed *in vitro* (Reid & Patrick, 1984). It is interesting that populations of *B. fragilis* NCTC 9343 from the 0–20% and 60–80% interfaces were resistant to killing by 20% normal human serum whereas *B. ovatus* ATCC 8483, which lacked an EDL in the absence of RR stain, was susceptible (Reid, 1983). Cells possessing an EDL were phagocytosed *in vitro*, but these layers (Figs 7 and 8) cannot be related to those observed on cells grown *in vitro* (e.g. Fig. 5) as material from the mouse may adhere to the bacterial surface.

Clearly it is important to determine the role of the ‘classical’ capsule of *Bacteroides* species (defined by wet India ink negative staining) in the pathogenicity of these organisms and also its relationship to the EDL observed by EM. Immunoelectron and immunofluorescence microscopy with monoclonal antibodies indicate that *B. fragilis* populations are antigenically heterogeneous (Reid *et al.*, 1985) and a monoclonal antibody with an epitope which is enriched in cells from the 20–40% interface has been characterized (unpublished result). It therefore seems that structural differences observed by EM are reflected by differences in antigenicity.

The authors thank Dr Evelyn Dermott for advice and encouragement during the preparation of this manuscript.
REFERENCES


