THE PATHOGENESIS OF MURINE INTRA-ABDOMINAL ABSCESSES: ULTRASTRUCTURAL AND QUANTITATIVE STUDIES

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ABSTRACT

A murine model of intra-abdominal (IA) abscess formation was used to study the interaction of murine strains of bacteria with peritoneal neutrophils. The intraperitoneal (IP) inoculation of non-immune mice with mixtures containing either $5x10^8$ Bacteroides fragilis or Bacteroides vulgatus combined with $1x10^6$ Escherichia coli and 1 mg of bran as a potentiating agent induced abscess formation after three days. Ten weeks after the IP inoculation of mice with B. fragilis, E. coli and bran IA abscesses containing viable bacteria at concentrations similar to those in the inoculum persisted in 71% of the mice.

During the first 24 hrs of infection B. fragilis and B. vulgatus were readily phagocytosed by neutrophils and some macrophages in the murine peritoneal cavity. However, after the first 4.5 hrs of infection, there were significantly more viable intracellular B. fragilis than B. vulgatus. Furthermore, after up to 24 hrs of phagocytosis in vivo, B. fragilis was more resistant than B. vulgatus to killing when the leukocytes were incubated with normal serum (NS) in vitro. This suggests that B. fragilis persists in IA abscesses because of its resistance to the bactericidal mechanisms of neutrophils after phagocytosis. To test this hypothesis, the extent of fusion of peroxidase-labelled primary granules with neutrophil phagosomes containing B. fragilis or B. vulgatus was examined by electron microscopy. After the in vivo phagocytosis of either B. fragilis or B. vulgatus, primary granules had fused with some bacteria-containing phagosomes of neutrophils. Intact primary granules were also visible in the neutrophils' cytoplasm. However, more damaged intracellular B. vulgatus than B. fragilis were observed. This was consistent with the significant reduction in the number of viable B. vulgatus in IA abscesses three weeks after the IP inoculation of mice with B. vulgatus, E. coli and bran.

The E. coli strain was encapsulated and was relatively resistant to in vitro phagocytosis in either NS or NS and immune serum (IS). This may be important in the persistence of the

infection. E. coli, either alive or killed, had no detectable effect on the phagocytic killing of B. fragilis in vitro. Although capsules were also detected on the B. fragilis and B. vulgatus strains by electron microscopy, both were readily phagocytosed in the presence of NS (a source of complement). In vitro phagocytic killing of B. vulgatus, at a ratio of one bacterium per ten peritoneal leukocytes, occurred in NS alone, whereas the maximal phagocytic killing of B. fragilis and E. coli required NS and IS. Phagocytic killing of B. fragilis and E. coli was significantly reduced in anaerobic conditions.

In the presence or absence of on-going phagocytosis, at a ratio of 100 bacteria per peritoneal leukocyte, a proportion of intracellular B. fragilis resisted the bactericidal mechanisms of neutrophils for at least 2 hrs in the in vitro assays. Intracellular B. fragilis were more resistant to ultrastructural damage than were B. vulgatus in the presence of on-going phagocytosis. Ingested B. fragilis were located within the phagosomes of neutrophils, and there was evidence of primary granule fusion with 15% and 13% of these phagosomes in NS and NS plus IS respectively. More phagocytic killing occurred in IS because, although the addition of IS to NS did not alter the percentage of phagocytes with intracellular bacteria, it did result in the phagocytosis of a greater number of bacteria per neutrophil. This resulted in more phagosomes per neutrophil in NS and IS, although the number of bacteria per phagosome and the proportion of peroxidase-positive phagosomes were similar to those in NS alone. Consequently, overall more bacteria were exposed to granule contents in NS and IS and more were killed by the peritoneal neutrophils than in NS alone. However, the small proportions of peroxidase-positive phagosomes in either NS or NS and IS, plus the survival of a greater proportion of B. fragilis when exposed to neutrophils at high vs low ratios of bacteria to peritoneal leukocytes, suggests that the fusion of an insufficient number of primary granules may influence the ability of neutrophils to kill bacteria readily phagocytosed at high ratios of bacteria to leukocytes.

A role for extracellular NS in the process of phagosome-granule fusion within neutrophils was demonstrated. After the phagocytosis of pre-opsonized B. fragilis in the presence of NS, which supported intracellular killing of the majority of the bacteria, few peroxidase-positive or peroxidase-negative granules were seen in the cytoplasm of neutrophils, indicating that phagosome-granule fusion had occurred. In contrast, in either NS heated to inactivate complement or the absence of NS, which did not support intracellular killing of B. fragilis, many intact granules were visible in the cytoplasm of neutrophils.

Bran was an essential component of the abscess-inducing mixture. In vitro, the phagocytic killing of B. fragilis and E. coli was reduced in the presence of bran. This effect of bran was observed with pre-opsonized bacteria in NS and suggests that bran affects the serum components, probably complement, necessary for the stimulation of intracellular killing.

After 120 mins of in vitro phagocytosis, the coalescence of phagosomes containing B. fragilis was evident in some neutrophils. The disintegration of the membranes of some necrotic neutrophils released bacteria from the phagosomes. Intracellular killing assays indicated that 20-40% of B. fragilis were viable at this time. Furthermore, bacteria were located in extracellular and intracellular sites within the abscesses. Thus, it is suggested that the establishment of a cycle of phagocytosis, limited intracellular killing due to insufficient fusion of primary granules with phagosomes in the presence of large numbers of bacteria, a situation compounded by the low levels of extracellular NS components, followed by release of the bacteria and limited bacterial replication, enabled the survival of bacteria in IA abscesses in mice.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university, and that to the best of my knowledge and belief, it does not contain any material previously published or written by another person, except where due reference is made in the text.

Lesley Hampton

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ABBREVIATIONS

AIM	An abscess-inducing mixture containing: 1 mg bran, $5x10^8$ colony forming units of *Bacteroides fragilis*, $1x10^6$ colony forming units of *Escherichia coli* in 0.05 ml of RPMI-1640 medium
BHIB	Brain heart infusion broth
cfu	Colony forming units
EM	Electron microscopy
FCS	Foetal calf serum
HNS	Heat-inactivated normal serum
IA	Intra-abdominal
IP	Intraperitoneal
IS	Heat-inactivated immune serum
LPS	Lipopolysaccharide
NS	Fresh normal serum
PA-TCH-SP	Periodate-thiocarbohydrazide-silver proteinate
PBS	Phosphate-buffered saline
SC	Subcutaneous
SD	Standard deviation
WC	Wilkins Chalgren

CHAPTER 1

INTRODUCTION

Bacteroides fragilis is the most frequently isolated anaerobic species in many infectious processes, including intra-abdominal (IA) abscesses (Altemeier et al., 1973; Bartlett, 1981; McGowan & Gorbach, 1981; Haase & Harding, 1985). These IA infections are often mixed and Escherichia coli is also commonly present (Bartlett, 1981). Other undefined components of intestinal contents potentiate the infection, and this is illustrated by the need for autoclaved intestinal contents as a potentiating agent in animal models of IA sepsis (Onderdonk et al., 1976; Joiner et al., 1980b; Nulsen et al., 1983).

Bacteria which contaminate the peritoneal cavity are removed by opsonization and phagocytic killing, lymphatic clearance of phagocytosed and non-phagocytosed bacteria and trapping of the bacteria either by fibrin or within adhesions between visceral surfaces (Dunn et al., 1985b). A role for cell-mediated immunity in both protection against (Onderdonk et al., 1982; Shapiro et al., 1982) and development of (Nulsen et al., 1986; Shapiro et al., 1986) IA abscesses in animal models has been demonstrated. Inoculation of the peritoneal cavity of animals with bacteria evokes an acute inflammatory response with the emigration of many neutrophils into the peritoneal cavity (Bartlett, 1981). Neutrophils are the major cell type involved in the development of abscesses (Hurley, 1983).

The ability of neutrophils to phagocytose and kill B. fragilis and E. coli has been studied in vitro. Members of the genus Bacteroides are opsonized in human normal serum (NS; Bjornson & Bjornson, 1978; Tofte et al., 1980; Joiner et al., 1981) and murine NS (Ellis & Barrett, 1982; Finlay-Jones et al., unpublished). Activation of both the alternative and classical complement pathways and the presence of specific immunoglobulins facilitates opsonization and phagocytic killing. Opsonins are present in peritoneal exudate (Bartlett, 1981) but their availability in abscesses is probably restricted (Gordon et al.,

1988). B. fragilis, in association with E. coli, persisted for at least 10 weeks in IA abscesses in mice (Nulsen et al., 1983). Furthermore, neutrophils from murine IA abscesses were unable to kill in vitro, in the presence of NS, the B. fragilis and E. coli they had phagocytosed in vivo (Hart et al., 1986b). Thus, intracellular killing of bacteria is not an inevitable consequence of their phagocytosis by neutrophils in vivo.

The interaction of B. fragilis with neutrophils in a mixed population of peritoneal leukocytes is the main aspect of abscess pathogenesis studied in this thesis. A combination of quantitative and ultrastructural techniques is applied both in vivo (Chapter 3) and in defined in vitro conditions (Chapter 4). The hypothesis that forms the basis of this thesis is that bacteria persist in IA abscesses because of their resistance to the bactericidal mechanisms of neutrophils after they have been phagocytosed.

The remainder of this chapter is a review of the literature on the pathogenesis of IA sepsis and the host defences available to quell the infection. The current information on the phagocytic process is reviewed as well as in vitro studies on the phagocytosis of Bacteroides species.

1.1 HUMAN INTRA-ABDOMINAL ABSCESSES

IA sepsis followed by IA abscess development in humans is representative of a mixed infection involving synergistic interactions between anaerobes and facultative anaerobes such as B. fragilis and E. coli (McGowan & Gorbach, 1981). IA infections are considered to be life-threatening (Haase & Harding, 1985). A breach of the colonic lumen due to intestinal injury, disease or surgery is the usual source of the infecting bacteria (Bartlett, 1981). Abscess development serves to limit

the spread of infection in the peritoneal cavity but the abscesses are often difficult to detect and treat (Haase & Harding, 1985). A lack of early treatment can compromise the efficacy of IA abscess treatment. Treatment usually involves the use of antimicrobial agents and surgery to remove infected fluids, foreign material and necrotic debris (Haase & Harding, 1985). However, opinions still differ on the best management of IA sepsis (Russell, 1987).

1.2 VIRULENCE OF B. FRAGILIS IN ANIMAL MODELS OF INTRA-ABDOMINAL SEPSIS

1.2.1 Animal Models of IA Sepsis

Several animal models have been used to study aspects of the development of IA sepsis using either SC (Joiner et al., 1980b; Verweij-van Vught et al., 1985) or intraperitoneal (IP; Onderdonk et al., 1977; McConville et al., 1981; Shapiro et al., 1982; Nulsen et al., 1983, Patrick et al., 1984; Rotstein et al., 1985b; Thadepalli et al., 1986) sites of infection. Weinstein et al. (1974) surgically implanted gelatin capsules containing the colonic contents from meat-fed rats plus $BaSO_4$ into the peritoneal cavities of rats and observed the development of IA sepsis consisted of two phases. An initial period of peritonitis associated with mortality was followed by the development of IA abscesses. Subsequently it was shown that the presence of E. coli was associated with the initial lethal phase, whereas B. fragilis, Fusobacterium, E. coli and enterococci were the main isolates from IA abscesses (Onderdonk et al., 1974). In a later study these anaerobic and facultatively anaerobic organisms were implanted IP into rats, either singly or in all possible pairings, plus the potentiating agents $BaSO_4$ and autoclaved colonic contents (Onderdonk et al., 1976). The results suggested a synergistic relationship existed between the

anaerobes and the facultative anaerobes. This rat model of IA sepsis has subsequently been used to study the pathogenicity of B. fragilis (reviewed by Onderdonk et al., 1984).

Surgical implantation of abscess-inducing mixtures has also been a feature of other animal models of IA sepsis. Patrick et al (1984) implanted chambers made from 1 ml syringe barrels, and filled with encapsulated or non-encapsulated strains of B. fragilis, into the peritoneal cavities of mice in order to study the growth and survival of the strains. Fibrin clots containing B. fragilis and E. coli were surgically implanted into the peritoneal cavities of rats and the synergy between the two organisms was investigated (Rotstein et al., 1985b).

The model of IA abscess formation in mice developed by Nulsen et al. (1983), and used in experiments described in this thesis, does not require surgical implantation of the bacteria and potentiating agent. The use of bran as a potentiating agent avoids the use of autoclaved caecal and colonic contents which do not have a well-characterized composition. Quantification of abscess development is possible by measuring the weight, incidence and bacterial content of the abscesses. In this model the IA abscesses formed resembled human IA abscesses in terms of abscess development, bacteriology and histology.

1.2.2 Encapsulation of B. fragilis

The microbial surface plays a role in the pathogenicity of many organisms (Smith, 1977; Costerton et al., 1981; Easmon et al., 1983). Costerton et al. (1981) used the term glycocalyx for any polysaccharide-containing bacterial surface structure external to the outer membrane of gram-negative bacteria or to the peptidoglycan layer of gram-

positive bacteria. Capsules may be described as micro- or macro-capsules depending on the amount of capsular material present. Penn (1983) recommends capsules should be defined functionally, morphologically and biochemically before being accepted as true capsules. Whether or not B. fragilis and other Bacteroides species possess capsules and the role of the capsule in bacterial virulence have been the subjects of several studies.

(a) Demonstration of capsules on Bacteroides species

Encapsulation of B. fragilis and other Bacteroides species has been studied by either light microscopy (Babb & Cummins, 1978; Patrick & Reid, 1983), electron microscopy (Kasper, 1976) or both microscopy techniques (Kasper et al., 1977; Woo et al., 1979; Bjornson et al., 1983, Strohm et al., 1983, Lambe et al., 1984; Patrick et al., 1984, Patrick et al., 1986). Initially only B. fragilis strains were reported to be encapsulated (Kasper et al., 1977). However, it has since been demonstrated that the possession of a capsule is not restricted to strains of B. fragilis. Capsules have been demonstrated on strains of B. vulgatus (Babb & Cummins, 1978; Bjornson et al., 1983; Strohm et al., 1983), Bacteroides thetaiotaomicron (Babb & Cummins, 1978; Bjornson et al., 1983; Strohm et al., 1983, Lambe et al., 1984), Bacteroides ovatus (Babb & Cummins, 1978; Strohm et al., 1983, Patrick et al., 1986), Bacteroides melaninogenicus (Woo et al., 1979), Bacteroides asaccharolyticus (Woo et al., 1979; Lambe et al., 1984), Bacteroides intermedius (Woo et al., 1979; Lambe et al., 1984) and Bacteroides corporis (Lambe et al., 1984).

Electron microscopy has revealed a range of capsular structures of varying thickness amongst Bacteroides species. Some extracellular polysaccharides detected by

electron microscopy may not be visible under the light microscope (Babb & Cummins, 1978; Strohm et al., 1983). However, both macro- and micro-capsules are important structures to consider in relationship to the pathogenicity of Bacteroides species since they form the external surfaces of the bacteria (Babb & Cummins, 1978).

(b) Encapsulation of Bacteroides species and virulence

The capsule of B. fragilis has been implicated as an important virulence factor in abscess development in a rat model of IA sepsis. An encapsulated B. fragilis strain (ATCC 23745), either alone or with a facultative anaerobe, induced abscess formation whereas, non-encapsulated Bacteroides species could induce abscesses to form only when combined with a facultative anaerobe. Furthermore, capsular polysaccharide extracted from B. fragilis caused abscess formation (Onderdonk et al., 1977). Brook and Walker (1983, 1984) compared the ability of encapsulated anaerobes with that of non-encapsulated anaerobes to induce SC abscess formation in mice. Encapsulated Bacteroides species were significant pathogens in their murine model.

The encapsulated B. fragilis strain ATCC 23745 is reported to be resistant to phagocytosis by neutrophils in vitro (Simon et al., 1982). In this study, more capsule was observed around animal-passaged B. fragilis than laboratory-passaged B. fragilis. The presence of a capsule correlated with the increased survival of animal-passaged B. fragilis in rabbit IP chambers. Patrick et al. (1984) examined the survival in vivo of homogeneous populations of either encapsulated or non-encapsulated B. fragilis strains obtained after application of cultures to Percoll gradients. In multiple in vivo experiments

exponential growth of encapsulated and non-encapsulated B. fragilis ATCC 23745 did not occur, although in some experiments growth occurred after prolonged incubation. In contrast, non-encapsulated populations of two other B. fragilis strains grew as well in vivo as their encapsulated counterparts.

In general, it is accepted that strains of B. fragilis and other Bacteroides species possess capsules. However, some studies have found no correlation between encapsulation of the bacteria and virulence (Babb & Cummins, 1978; Verweij-van Vught et al., 1986), while others claim that capsular polysaccharides of B. fragilis strains may possess unique virulence properties (Strohm et al., 1983; Kasper et al., 1984). In vitro studies on the virulence of encapsulated B. fragilis in relation to phagocytosis by neutrophils will be discussed in Section 1.5.5.

1.2.3 Bacterial Synergy

Synergistic relationships between anaerobic bacteria and facultatively anaerobic bacteria have been demonstrated repeatedly in animal models of IA sepsis (Table 1.1) and have been the subject of a recent review (Rotstein et al., 1985c). However, the mechanism(s) by which synergy is achieved remains to be elucidated.

Reznikov et al. (1981) found that B. fragilis strains had no effect on the clearance of E. coli from the peritoneal cavities of mice. Another group found that B. fragilis did not compete with E. coli for clearance or opsonization and phagocytosis within the peritoneal cavity of the rat (Dunn et al., 1985a).

Table 1.1: Bacterial Synergy in Animal Models of IA Sepsis[a]

Bacterial Inoculum	Animal Model	Expression of Synergy	Reference
Organisms from peritonitis secondary to perforated appendix	Guinea pig: SC and IP	Cellulitis & mortality	Altemeier (1942)
Bacterial isolates ± *B. melaninogenicus* from human faeces	Guinea pig: SC	Mortality, cellulitis & abscess	Socransky & Gibbons (1965)
B. fragilis + *Fusobacterium necrophorum*; *F. necrophorum* + *Fusobacterium nucleatum*	Mouse: IP with mucin	Mortality & liver abscess	Hill *et al.* (1974)
Rat caecal contents	Rat: IP with $BaSO_4$ in gelatin capsules	Abscess	Onderdonk *et al.* (1974)
Rat caecal contents	Rat: IP with $BaSO_4$ in gelatin capsules	Abscess	Weinstein *et al.* (1974)
E. coli + *B. fragilis*	Rabbit: IV[b] injection	Mortality & liver abscess	Nielsen *et al.* (1976)
E. coli, *B. fragilis*, enterococcus, *Fusobacterium varium*	Rat: IP with $BaSO_4$ & autoclaved colonic contents in gelatin capsules	Abscess	Onderdonk *et al.* (1976)
F. necrophorum ± *B. fragilis*, *B. vulgatus* or *B. ovatus*	Mouse: SC	Mortality	Wilkins *et al.* (1977)

Table 1.1 cont'd:

Bacterial Inoculum	Animal Model	Expression of Synergy	Reference
E. coli + *B. fragilis*	Guinea pig: SC	Abscess	Kelly (1978)
B. melaninogenicus + *F. necrophorum*	Mouse: IP	Abscess & mortality	McCallum *et al.* (1982)
E. coli + *B. fragilis*	Guinea pig: SC	Abscess & mortality	Shapiro & Sacks (1982)
F. necrophorum + *B. melaninogenicus*	Mouse: IP	Abscess & mortality	McCallum *et al.* (1983)
E. coli + *B. fragilis*	Mouse: IP with autoclaved caecal & colonic contents	Abscess	Nulsen *et al.* (1983)
E. coli + *B. fragilis*	Mouse: IV injection	Mortality & increased survival of bacteria	Rodloff & Hahn (1984)
E. coli + *B. fragilis*	Rat: fibrin-clot model	Mortality	Rotstein *et al.* (1985b)
E. coli + *B. fragilis*	Mouse: SC	Abscess	Verweij-van Vught *et al.* (1985)
E. coli + *B. fragilis*; *E. coli* + *B. vulgatus*	Mouse: SC	Abscess	Verweij-van Vught *et al.* (1986)

[a] Based on Rotstein *et al.* (1985c)
[b] IV, intravenous

Recent studies have focused on the role of E. coli in synergy with anaerobic bacteria. In an in vitro study Hagen et al. (1982) examined the effect of E. coli on the growth of B. fragilis in conditions not suitable for growth of the anaerobe. It was suggested that E. coli releases growth-stimulating factors for B. fragilis because the growth of B. fragilis was supported by media which previously contained growing E. coli. Although the presence of E. coli reduced the level of oxygen in the media that alone was not sufficient to support the growth of B. fragilis. Nulsen et al. (1983) showed that although the abscesses were smaller and less persistent, B. fragilis was able to induce IA abscess formation in the absence of E. coli in mice. This also suggests that synergy between the two organisms observed in vivo is not due solely to the creation of an anaerobic environment by the facultative anaerobe.

Rodloff and Hahn (1984) inoculated mice with either B. fragilis, E. coli or combinations of both organisms and measured the number of bacteria surviving in the livers and kidneys nine days after inoculation. Synergy between the bacteria, expressed as either increased survival of bacteria in the organs or as a lethal effect, was only apparent if the dose of E. coli inoculated by itself was high enough to cause the death of some infected mice. However, the non-specific effect of large numbers of bacteria on the ability of the reticulo-endothelial system to eradicate the bacteria was not discussed by the authors. Synergy was manifested as a lethal effect on rats following the IP implantation of a fibrin clot containing B. fragilis and E. coli (Rotstein et al., 1985b). These results supported the notion that a heat-stable factor produced by B. fragilis serves to increase the lethal effects of E. coli.

In an SC model of abscess formation in mice, only one of two E. coli strains was capable of establishing a synergistic relationship with two B. fragilis strains (Verweij-van Vught et al., 1985). When synergy between B. fragilis and E. coli was observed, there was reduced clearance of both organisms. The results of their study indicate synergy is dependent on properties of individual E. coli strains.

Synergistic relationships between E. coli and either B. fragilis or B. vulgatus have also been compared in the murine model of SC abscess formation (Verweij-van Vught et al., 1986). B. vulgatus was cleared more rapidly from SC sites than was B. fragilis when the anaerobes were inoculated without E. coli. However, depending on the inoculation of sufficient numbers of E. coli, either B. fragilis or B. vulgatus when combined with E. coli caused abscesses to form. The authors conclude that both B. fragilis and B. vulgatus can act synergistically with E. coli but the inherent virulence of the anaerobe and its ability to be maintained in sufficient numbers in vivo are important determinants of the outcome of the infection.

Studies have not conclusively established a role for the capsular polysaccharide of B. fragilis in the synergy observed between B. fragilis and E. coli in animal models (Rodloff & Hahn, 1984; Rotstein et al., 1985c). However, one proposed mechanism of synergy is the inhibition by the anaerobes of the phagocytic killing of the facultative anaerobes. In vitro studies of this are discussed in Section 1.5.4, but one group examined this in vivo. Peritoneal macrophages from mice, inoculated intravenously 6-12 hrs earlier with encapsulated B. fragilis, were inhibited in their phagocytic activity (Rodloff et al., 1986). This effect did not appear to be due to overloading of the macrophages with bacteria. B. fragilis with thinner

capsules, other bacteria or latex particles were either not inhibitory of macrophage phagocytosis or less effective. The authors suggest that this effect of encapsulated B. fragilis on macrophage phagocytosis may be involved in the synergy between the anaerobe and facultative anaerobes.

1.2.4 Other Virulence Factors of B. fragilis

(a) Endotoxin

The endotoxic lipopolysaccharide (LPS) of B. fragilis is structurally different from the endotoxins of members of the Enterobacteriaceae. It contains only small amounts of 3-hydroxytetradecanoic acid which is a major fatty acid in the LPS of members of the Enterobacteriaceae (Weintraub et al., 1985). Moreover, the endotoxin of B. fragilis is not as biologically active as enterobacterial endotoxin (Hofstad & Sveen, 1979; Kasper et al., 1979; Hofstad, 1984; Salyers, 1984). However, one group found that the activity of B. fragilis endotoxin, measured only by the local Schwartzman reaction, was similar to that of endotoxin from an E. coli strain (Meisel-Mikolajczyk et al., 1984). Bacteroides LPS can activate complement and generate C5a, which is chemotactic for neutrophils (Hofstad, 1984). However, in a rat model of IA sepsis, the LPS of two B. fragilis strains did not promote abscess formation (Kasper et al., 1984).

(b) Enzymes

B. fragilis strains synthesize a range of extracellular and cell-bound enzymes. These include collagenase, hyaluronidase, chondroitin sulphatase, fibrinolysin, neuraminidase, heparinase and DNase (Hofstad, 1984). The roles of the enzymes in the virulence of B. fragilis are not fully understood but some enzymes could be predicted to contribute to the suppuration associated with abscess

formation. The production of superoxide dismutase is related to the oxygen tolerance of B. fragilis and possibly to the ability of the organism to survive in tissues until anaerobic conditions develop (Tally et al., 1977; Gregory et al., 1978; Rolfe et al., 1978).

(c) Adherence

Pruzzo et al. (1984) examined the ability of non-encapsulated B. fragilis strains to adhere to human epithelial cells in response to a study by Onderdonk et al. (1978), which showed the capsule of B. fragilis promotes adherence to rat peritoneal mesothelium. Those non-encapsulated strains of B. fragilis which adhered to epithelial cells and haemagglutinated guinea pig and human erythrocytes possessed pili. However, the role of pili as virulence factors is unclear since piliated bacteria were more susceptible to phagocytosis and killing by human neutrophils in vitro than were non-piliated bacteria.

(d) Succinic acid

Succinic acid, a short-chain fatty acid produced by B. fragilis and other Bacteroides species, has been implicated in the virulence of the bacteria. Succinic acid inhibits the phagocytic killing of E. coli and the production of chemiluminescence by neutrophils in vitro at an acidic but not neutral pH (Rotstein et al., 1985a). The reduction of the intracellular pH of neutrophils by succinic acid was partly responsible for the impairment of the neutrophil respiratory burst (Rotstein et al., 1987). High concentrations of succinic acid and other short-chain fatty acids are present in infections containing Bacteroides species (Gorbach et al., 1976). The pH of pus from experimental abscesses ranges from acid to neutral (Hays & Mandell, 1974). It has been suggested that in vivo, in the presence of a reduced

extracellular pH, succinic acid could further reduce the intracellular pH of the neutrophils, thereby causing inhibition of neutrophil functions (Rotstein et al., 1987).

(e) Response to Iron Restriction

Iron is required by bacteria for growth and bacteria possess mechanisms by which they can acquire iron in competition with their hosts (Finkelstein et al., 1983). Recently it was found that B. fragilis, unlike B. vulgatus, produces several outer membrane proteins in response to iron restriction (Otto et al., 1988). The authors suggest that these proteins may contribute to the virulence of B. fragilis.

(f) Enterotoxin

Myers et al. (1987) reported the isolation of enterotoxin-producing strains of B. fragilis from 8/44 humans with diarrhoea. In seven cases this was the sole enteric pathogen isolated. In a rabbit model, enterotoxigenic B. fragilis caused moderate to severe necrotizing colitis and $5x10^3$ bacteria were sufficient to cause death of the animal. Like other enteric pathogens, enterotoxigenic strains of B. fragilis have also been isolated from healthy humans. However, the contribution of an enterotoxin to the virulence of B. fragilis in abscess formation has not been investigated.

1.3 HOST DEFENCES IN THE PERITONEAL CAVITY

The host defence mechanisms available to remove bacteria from the peritoneal cavity are:

(i) removal by diaphragmatic lymphatics;

(ii) opsonization and phagocytic killing; and

(iii) trapping of the bacteria, either by fibrin or within adhesions between visceral surfaces (Dunn et al., 1985b).

The effectiveness of these mechanisms will be influenced by the number of bacteria infecting the peritoneal cavity and the presence of adjuvants or potentiating agents.

1.3.1 Lymphatic Clearance

Lymphatic drainage units are found along the peritoneal surface of the muscular portion of the diaphragm. They are drained by lymphatic vessels which traverse the diaphragm and eventually lymphatic fluid enters the bloodstream via thoracic ducts (Tsilibary & Wissig, 1977; 1987). Clearance of bacteria is rapid as bacteraemia occurs within minutes after inoculation of bacteria into the peritoneal cavity (Bartlett, 1981).

The importance of peritoneal clearance has been emphasized in studies of experimental peritonitis. Jennings et al. (1980) examined the response of the defence mechanisms of the rat in three types of experimental peritonitis. In non-fatal peritonitis caused by an avirulent strain of E. coli, bacteria were readily removed from the peritoneal cavity

within 4 hrs of infection. However, in fatal peritonitis induced by inoculation of virulent E. coli, with or without haemoglobin, clearance was impaired and the bacteria replicated.

Thoracic duct ligation impeded the normally rapid clearance of radiolabelled non-viable E. coli from the rat peritoneal cavity (Dunn et al., 1985b). In rats with intact thoracic ducts, more E. coli were removed by clearance from the peritoneal cavity than by phagocytosis within the peritoneal cavity, irrespective of the number of E. coli inoculated. This study did not distinguish between peritoneal clearance of phagocytosed and non-phagocytosed bacteria. Lymphatic clearance continued after maximal phagocytosis had occurred. Similar results were obtained with radiolabelled non-viable B. fragilis.

In an experimental model of peritonitis in pigs, B. fragilis and E. coli were rapidly cleared from the peritoneal cavity in the initial 3 hrs of infection (Skau et al., 1986). However, this was followed by stabilization of the remaining low numbers of bacteria in the peritoneal cavity. When a second dose of bacteria was inoculated 6 hrs after the first dose, a similar clearance pattern was obtained. The authors suggest that although clearance of the bacteria from the peritoneal cavity was incomplete, the exhaustion of any significant component of the local defences was not responsible.

1.3.2 Humoral Defence Mechanisms

Serum from healthy humans contains antibodies to members of the Bacteroidaceae (Quick et al., 1972). Elevated titres of antibody are found in serum from individuals with infections caused by bacteria belonging to the Bacteroidaceae (Quick et

al., 1972; Hofstad, 1979).

Circulating antibodies specific for B. fragilis did not provide protection from IA abscesses in mice (Nulsen, 1982; Shapiro et al., 1982). Although rats actively immunized with B. fragilis capsular polysaccharide were protected to a significant extent from the formation of IA abscesses (Kasper et al., 1979), the passive transfer of hyperimmune serum from immunized rats to non-immune rats did not confer protection (Onderdonk et al., 1982). The transfer of spleen cells from immune rats to non-immune rats did prevent IA abscess development (Onderdonk et al., 1982).

The ability of neutrophils in the murine peritoneal cavity to phagocytose and kill E. coli was augmented by the presence of complement and specific antibodies, especially those antibodies directed against the K antigens of the organism (Ahlstedt, 1983). Mice, which either survived a sub-lethal IP infection with E. coli or were pre-treated with E. coli endotoxin, showed some resistance to a subsequent, otherwise lethal, experimental E. coli peritonitis (Vuopio-Varkila & Mäkelä, 1988). Antibody-mediated opsonization and phagocytic killing of the E. coli played a significant contribution in the removal of the bacteria from the peritoneal cavity.

B. fragilis and other Bacteroides species can activate both the alternative (Joiner et al., 1981) and classical (Tofte et al., 1980) pathways of the complement system in vitro. Strains of B. fragilis (Joiner et al., 1981; Bjornson et al., 1983; Reid & Patrick, 1984; Rotimi & Eke, 1984), Bacteroides distasonis (Joiner et al., 1981; Bjornson et al., 1983), B. ovatus (Joiner et al., 1981; Bjornson et al., 1983), B. vulgatus (Bjornson et al., 1983) and B. thetaiotaomicron (Bjornson et al., 1983) have been shown to be resistant to the bactericidal action of human NS.

Joiner et al. (1980a) studied the formation of SC abscesses by B. fragilis in normal mice, C5-deficient mice and mice depleted of the alternative complement pathway by cobra venom factor. Mice pre-treated with cobra venom factor had larger abscesses than untreated mice. Abscesses from C5-deficient mice and normal mice were similar in size.

In vitro studies on the role of complement in host resistance against members of the Bacteroidaceae have been reviewed (Bjornson, 1984), but the importance of the bactericidal action of complement and the role of complement in the opsonization and phagocytic killing of Bacteroides species in vivo remains to be conclusively established.

1.3.3 Phagocytosis

Leukocytes from the unstimulated peritoneal cavity of the mouse are composed of 54% resident macrophages, 25% lymphocytes, 9% monocytes, 6% mast cells, 4% eosinophils and less than 1% neutrophils (Daems, 1980). Resident murine peritoneal macrophages are reported to phagocytose particles at a slower rate than do exudate peritoneal macrophages (Papadimitriou & van Bruggen, 1982). A feature of infection of the peritoneal cavity with bacteria is the continuous influx of neutrophils. The number of neutrophils entering the site of infection is influenced by the number and virulence of the bacteria (Bartlett, 1981).

Phagocytosis is reported to be the rate-limiting step in the removal of bacteria from the peritoneal cavity (Jenkin & Rowley, 1959; Cohn, 1962; Easmon & Glynn, 1976). However, in a rat model of E. coli peritonitis, phagocytes appeared to remove 33% of the bacterial inoculum within minutes (Dunn et al., 1985b). Phagocytosis of the bacteria was attributed to

the resident peritoneal macrophages since the influx of neutrophils had not then occurred. The authors suggested that when neutrophils arrive, they deal with those bacteria that have escaped the host defences already in operation in the peritoneal cavity. This role for neutrophils was not supported by the results of Freischlag et al. (1986). In their rabbit model of experimental peritonitis, neutrophils which had migrated into the peritoneal cavity were ineffective in promoting killing of the infecting bacteria. Furthermore, although the concentration of neutrophils in the peritoneal cavities of pigs increased after IP inoculation with E. coli and B. fragilis, a second dose of bacteria was not eliminated at a faster rate (Skau et al., 1986). Thus, some disagreement exists as to the contribution made by each of the different types of phagocytic cells to removal of the bacteria.

Those bacteria that are phagocytosed are subject to the potentially toxic environment of the neutrophil or macrophage phagosome. The fate of phagocytosed bacteria within the murine peritoneal cavity has been studied by electron microscopy (Iwata et al., 1978; Guo et al., 1986a; Guo et al., 1986b). After IP inoculation, a highly virulent strain of Staphylococcus aureus was more resistant to phagocytosis by macrophages than were a strain of low virulence and an avirulent strain (Iwata et al., 1978). After 24 hrs the highly virulent strain remained resistant to intracellular degradation once phagocytosed by macrophages, and some macrophages were degenerating. In contrast, the low-virulent and avirulent strains were readily degraded by the macrophages.

Virulent Salmonella typhimurium inoculated IP into mice were ingested by neutrophils and macrophages within 3 hrs of infection (Guo et al., 1986a). Intracellular bacteria were degraded by the phagocytes. Some phagocytes contained

bacteria showing various degrees of damage, which the authors suggested indicated the ability of the phagocytes to continue ingesting while degrading those bacteria already phagocytosed. Extracellular replication of some bacteria occurred. Opsonized S. typhimurium were more rapidly phagocytosed upon IP inoculation, but the rapid degradation of intracellular bacteria appeared to be independent of opsonic antibody (Guo et al., 1986b).

1.3.4 Cell-mediated Immunity

The role of cell-mediated immunity in the defence of the peritoneal cavity from infection with B. fragilis has been studied. Non-immune rats which received spleen cells from rats immunized with B. fragilis capsular polysaccharide were protected from IA abscess development when later challenged with B. fragilis and potentiating agents. The passive transfer of hyperimmune serum did not protect the non-immune rats (Onderdonk et al., 1982). These results indicate the existence of a T-cell mediated immune response.

In a murine model of IA abscess development, the T cells involved were not helper or killer T cells (Shapiro et al., 1982). A suppressor T cell factor has been described which protects mice from IA abscesses induced by B. fragilis (Zaleznik et al., 1985). Subsequently, it was shown that two types of $Ly\text{-}1^{-}2^{+}$ T cells are involved in the development of immunity to IA abscess formation by B. fragilis (Shapiro et al., 1986). The first type of T cell releases the suppressor T cell factor described by Zaleznik et al. (1985), which then interacts with the second type of $Ly\text{-}1^{-}2^{+}$ T cell. It is suggested that a suppressor-like T cell circuit functions in immunity to abscesses.

A role for T cells in abscess development has also been implicated. A T cell bearing Ly-1 and Ly-2 surface markers is necessary for the initiation of IA abscess development in mice (Shapiro et al., 1986). In a different murine model, T cells were found to be necessary for the formation of well-encapsulated abscesses in mice challenged with a mixture of B. fragilis, E. coli and a potentiating agent (Nulsen et al., 1986).

1.3.5 Abscesses

IA abscesses can be considered a host defence mechanism since the influx of neutrophils localizes the pyogenic bacteria at the site of the infection. Liquefactive necrosis occurs and the bacteria within the pus become enveloped by vascularized granulation tissue as the abscess ages (Hurley, 1983).

The primary granules of neutrophils contain acid hydrolases, neutral proteases, cationic proteins, myeloperoxidase, lysozyme and acid mucopolysaccharide. Lysozyme, lactoferrin, vitamin B_{12}-binding protein, collagenase and acidic proteins are found in the secondary granules. Granule contents may be released upon phagocytosis or due to death of the neutrophil. Neutrophils are also able to actively secrete the contents of their secondary granules extracellularly in vivo but at a site of inflammation, there is also some extracellular degranulation of the primary granules. The granule constituents released contribute to the role of the neutrophil in host defence by providing antimicrobial substances extracellularly, regulating the acute inflammatory response and by influencing the function of other inflammatory leukocytes (Wright, 1982). Substances released by neutrophils may also aid the process of liquefactive necrosis.

Defensins are antimicrobial and cytotoxic cationic proteins produced by neutrophils (Ganz et al., 1985). They are released extracellularly and their release correlates with the release of the primary granule markers beta-glucuronidase and elastase. It is thought that the defensins may reach effective bactericidal or cytotoxic concentrations in neutrophil-rich exudates (Ganz, 1987).

Despite the presence of viable neutrophils and microbicidal substances in pus, bacteria frequently persist within abscesses (Murphy, 1976). The reduced pO_2 and redox potential within abscesses may affect the ability of neutrophils to kill bacteria (Hays & Mandell, 1974). Thus abscess formation is not a totally effective host defence mechanism and IA abscesses are potentially harmful (Haase & Harding, 1985).

1.4 PHAGOCYTOSIS

1.4.1 Methodology

Aspects of the phagocytic process, i.e. opsonization, attachment and ingestion followed by intracellular killing, have been investigated by a variety of in vitro methods. A variety of gram-negative and gram-positive bacterial species or other particles and a range of animals as sources of leukocytes and sera have been used. The metabolic status of the bacteria (Brown & Williams, 1985; Bortolussi et al., 1987) and recent history of the organisms (Simon et al., 1982) will influence their susceptibility to the bactericidal activity of the phagocytes.

Sources of leukocytes include humans (Leijh et al., 1979a & b), mice (Finlay-Jones et al., 1984), bovines (Mackie et al.,

1982a & b) and rabbits (Freischlag et al., 1986). Leukocytes from sites such as the peripheral blood vessels and the peritoneal cavity have been studied. Various methods have been tried in order to elicit leukocytes containing a high percentage of neutrophils from the peritoneal cavity of the mouse (Baron & Proctor, 1982). Mixed populations of peritoneal leukocytes (Finlay-Jones et al., 1984) and enriched populations of peritoneal neutrophils obtained by discontinuous Percoll density gradients (Hart et al., 1985) have been used in assays. Murine peritoneal neutrophils and peripheral blood neutrophils have been shown to be similar in their phagocytic activity (Hart et al., 1986b), in contrast to neutrophils from equivalent sites in rabbits (Freischlag et al., 1986).

Fresh NS, heat-inactivated NS (HNS) and sera deficient of complement components or immunoglobulins have been included in assays. The concentration of serum used in experiments will affect the opsonization (Tofte et al., 1980; Bjornson et al., 1983; Gordon et al., 1988), phagocytosis (Leijh et al., 1979b) and intracellular killing (Leijh et al., 1981) of bacteria. Sera from a variety of animal species also differ in the quantity and biological activity of complement components and immunoglobulins.

Ratios of leukocytes to bacteria will influence the outcome of phagocytosis (Simmons & Karnovsky, 1973; Clawson & Repine, 1976; Leijh et al., 1979b; Matheisz & Allen, 1979). The interactions of bacteria and leukocytes have been studied with leukocytes in suspension either in test-tubes (Leijh et al., 1979a & b; Finlay-Jones et al., 1984; Hart et al., 1985) or in the wells of microtitre plates (Mackie et al., 1982a & b; Knowles & Weston, 1984). Neutrophils and macrophages adherent to surfaces have also been used (Smith & Rommel, 1977; Menzel et al., 1978; Pantazis & Kniker, 1979; Raynor et al., 1981; Goldner et al., 1983).

Systems of ongoing phagocytosis where bacteria, leukocytes and serum are in continuous contact give an overall measure of the bactericidal activity of leukocytes as determined by viable counts (Finlay-Jones et al., 1984) and radioactive-labelling methods (Mackie et al., 1982b).

Ingestion of bacteria, as a process distinct from intracellular bactericidal events, has been measured microbiologically as a function of the extracellular disappearance of the bacteria (Leijh et al., 1979b), by flow cytometry (Bassøe & Bjerknes, 1985) and by fluorescence microscopy (Hed, 1977; Goldner et al., 1983; Heesemann & Laufs, 1985).

Intracellular killing of bacteria by neutrophils has been determined by viable counts after the removal of non-phagocytosed bacteria by either differential centrifugation (Leijh et al., 1980) or discontinuous Percoll density gradients (Hart et al., 1985). The susceptibility of bacteria to killing by extracts prepared from neutrophil granules has been used as an indication of the ability of neutrophils to function in aerobic and anaerobic conditions (Pruul et al., 1983; Wetherall et al., 1984; Hodinka & Modrzakowski, 1986). Fluorescence microscopy has been used to determine the viability of phagocytosed bacteria which is revealed by staining with the fluorescent dye acridine orange (Smith & Rommel, 1977; Pantazis & Kniker, 1979; Goldner et al., 1983).

Transmission electron microscopy has provided information on the intracellular location of bacteria (Rikihisa & Ito, 1979; 1983) and the fusion of granules with phagosomes (Hirsch, 1962, Zucker-Franklin & Hirsch, 1964; Armstrong & Hart, 1971; Bainton, 1973; Pryzwansky et al., 1979; Rikihisa & Ito, 1982; Horwitz, 1983; Riley & Robertson, 1984; Bertram et al., 1986, Frehel et al., 1986, Frehel & Rastogi, 1987).

Intracellular degradation of bacteria has been studied *in vitro* by transmission electron microscopy (Goodman *et al.*, 1956; Ayoub & White, 1969; De Voe *et al.*, 1973 a & b; Mackie *et al.*, 1982a, Riley & Robertson, 1984; Rozenberg-Arska *et al.*, 1985; Young *et al.*, 1985), flow cytometry (Bassøe & Bjerknes, 1985) and the radioactive labelling of bacterial components (Berto & Menzel, 1979; Thore *et al.*, 1985; Weiss *et al.*, 1987).

Due to the variety of methods available and the number of parameters to be considered in studies on the stages of the phagocytic process, a panoply of results is to be found in the published literature. The current state of knowledge with regard to opsonization, phagocytosis and intracellular killing of bacteria by leukocytes will be reviewed in the following sections. Recent published reviews on these subjects include Densen and Mandell (1980), Elsbach (1980), Root and Cohen (1981), Horwitz (1982), Ryter and de Chastellier (1983), Spitznagel (1983) plus Beaman and Beaman (1984). The published information on the interaction of *Bacteroides* species with leukocytes is discussed in Section 1.5.

1.4.2 Opsonization

Bacteria vary in their requirements for opsonization (Guckian *et al.*, 1978; Leist-Welsh & Bjornson, 1979). C3 fragments and IgG are the most important opsonins of bacteria in serum.

The four classes of IgG bind to antigenic determinants on the bacterial surface by the two antigen binding sites ($F(ab)_2$) of the immunoglobulin molecule, but only IgG_1 and IgG_3 bind to the Fc receptors of phagocytes (Griffin, 1977). Specific antibacterial antibodies are able to increase the rate of

complement activation (Joiner et al., 1984). The presence of both immunoglobulins and complement enhances the opsonization of encapsulated bacteria.

Specific antibody was necessary for the fixation of opsonic C3 to the surface of an encapsulated strain of E. coli (Horwitz & Silverstein, 1980). Wilkinson et al. (1979) found that although C3 was fixed to the cell wall of an encapsulated S. aureus strain, its presence was masked by the bacterial capsule and therefore, C3 was not opsonically active. Brown et al. (1983) demonstrated that C3b molecules deposited on the capsule of Streptococcus pneumoniae by anti-capsular antibodies were opsonically active, whereas C3b deposited on the organism's cell wall by anti-cell wall antibodies was not opsonic. Thus, the presence of specific anti-capsular antibodies enables the fixation of C3 fragments on the outermost surface of the organism where they can be recognized by the appropriate phagocyte receptors.

In the non-immune animal, complement is considered to be the major opsonin of bacteria (Frank, 1979) but its effectiveness may be reduced in some sites, e.g. abscesses, where opsonin concentrations may be low (Gordon et al., 1988).

Activation of the classical complement pathway is either by IgG and IgM or by components of gram-negative bacteria such as Lipid A and rough LPS in the absence of antibody. The cell walls of gram-positive bacteria and the LPS of gram-negative bacteria activate the alternative pathway (Joiner et al., 1984).

Activation of either the alternative or classical pathways generates the opsonic C3b. Verbrugh et al. (1979) showed that the amount of C3 fixed to bacterial surfaces correlates with the ability of the neutrophils to phagocytose the organisms. C3b bound to the bacterial surface can further

activate the complement system or be cleaved to generate the opsonic fragment C3bi which remains bound to the organism (Joiner et al., 1984). Cleavage of C3bi can occur but it is a slow process. Gordon et al. (1988) found that a significant amount of opsonic C3 bound to both gram-positive and gram-negative bacterial surfaces was C3bi.

Other opsonins of less importance include IgM, IgA, other complement fragments and C-reactive protein.

In general, opsonization of bacteria is influenced by the bacterial surface structure, the presence of antibody and the pathway of complement activation. The enhanced virulence of encapsulated bacteria is well documented and has been reviewed by Smith (1977), Densen and Mandell (1980), Costerton et al. (1981), Horwitz (1982) and Easmon et al. (1983).

1.4.3 Attachment and Ingestion

Neutrophils and macrophages have Fc receptors for the different classes and subclasses of immunoglobulins (Unkeless & Wright, 1984). They also possess the complement receptors CR1, CR2 and CR3 which bind C3b, C3d and C3bi respectively (Wright and Griffin, 1985).

The complement receptors of neutrophils have been shown to be mainly involved in the attachment of C3-opsonized particles, including bacteria, whereas the Fc receptors have a primary role in the ingestion of IgG-opsonized particles (Mantovani, 1975; Scribner & Fahrney, 1976). Hed and Stendahl (1982) have shown that C3b-promoted ingestion of particles by neutrophils occurs but to a lesser extent than the active ingestion of particles promoted via the Fc receptors. Similarly, on the macrophage surface, the Fc receptors are

intrinsically active, whereas the complement receptors are usually inactive and their activation is under physiologic regulation (Unkeless & Wright, 1984). A lymphokine and insoluble stimuli such as fibronectin can activate the complement receptors (Wright & Griffin, 1985). In addition, a phagocytosis-stimulating factor produced by neutrophils has been shown to enhance complement receptor-mediated phagocytosis by macrophages (Ishibashi & Yamashita, 1987). It is suggested that this factor could increase the involvement of macrophages in the initial stages of inflammation when the lymphokine is not available. Co-operation between Fc receptors and complement receptors is thought to be necessary for the ingestion of particles by macrophages (Griffin, 1982).

Griffin (1982) described the "zipper" mechanism for the phagocytosis of particles, including bacteria, by phagocytes. Pseudopodia are able to encircle the opsonized particle due to the rearrangement of actin filaments. The particle-bound opsonins bind to the appropriate receptors on the membrane of the phagocyte's pseudopodia. The progressive involvement of opsonins and phagocyte receptors leads to complete encirclement of the particle by the pseudopodia creating the phagosome.

Phagocytosis of non-opsonized bacteria by neutrophils and macrophages is possible. Speert et al. (1986) showed that the phagocytosis of some strains of Pseudomonas aeruginosa in the absence of opsonins was promoted by bacterial piliation and hydrophobicity. Non-opsonized S. aureus were readily phagocytosed when the bacteria were adhering to glass or plastic surfaces (Devalon et al., 1987). Ofek and Sharon (1988) have reviewed lectinophagocytosis of bacteria. Bacteria carrying surface lectins bind to the appropriate carbohydrates on the surface of the phagocyte or conversely bacterial surface carbohydrates bind to lectins on the

phagocyte's membrane. Fibronectin functions as both a non-specific opsonin and as a stimulus for phagocytosis of bacteria, degradative products of tissue injury and cell debris (Marino & Spagnuolo, 1988).

1.4.4 Intracellular Events

The presence of serum components, either as particle-bound opsonins or free in the extracellular milieu, is necessary for the intracellular killing of bacteria by neutrophils, even after phagocytosis has occurred. Menzel et al. (1978) demonstrated increased killing of E. coli by human neutrophils when complement was activated by the antibody-coated bacteria. E. coli with surface-bound complement components were more susceptible to intracellular degradation by human neutrophils (Berto & Menzel, 1979). Murata et al. (1987) found that superoxide production by neutrophils was increased when zymosan particles opsonized with both IgG and C3b were phagocytosed compared with the phagocytosis of particles opsonized with either IgG or C3b.

Extracellular NS is essential for the intracellular killing of bacteria by human (Leijh et al., 1981) and murine (Hart et al., 1985) neutrophils. However, the involvement of either IgG or C3 fragments or both is still the subject of investigation.

Less killing of intracellular S. aureus and E. coli by human neutrophils occurred in HNS than in NS. The intracellular killing in HNS was attributed to the binding of IgG to Fc receptors on the neutrophils. The enhanced intracellular killing in NS was probably due to the interaction of C3 fragments with neutrophil complement receptors (Leijh et al., 1981).

In order to determine the importance of either the Fc or complement receptors of murine neutrophils on the intracellular killing of P. mirabilis and S. aureus, the appropriate monoclonal antibodies were used to block the Fc and complement receptors (Hart et al., 1986a). In murine HNS, the blocking of the Fc receptors did not decrease the intracellular killing of the bacteria compared with that in neutrophils with untreated Fc receptors. Functional C3 receptors were necessary for the intracellular killing of P. mirabilis in the presence of murine NS. A subsequent study measured the intracellular killing of P. mirabilis by murine neutrophils in NS, HNS, immune serum or heated immune serum (Hart et al., 1987). Intracellular killing was greatest in NS or immune serum and heating of these sera significantly reduced killing. Thus, it appears to be the extracellular heat-labile complement components of NS and immune serum which enhance the killing of P. mirabilis by murine neutrophils.

Some phagocytosed bacteria can resist the microbicidal mechanisms of neutrophils and macrophages. Inhibition of either the respiratory burst or degranulation and resistance to either oxygen-dependent killing or the toxicity of granule components are some of the "tactics" used by bacteria. Other bacteria avoid killing by escaping from the phagosome or by destruction of the phagocyte (Densen & Mandell, 1980).

Upon the phagocytosis of bacteria, the neutrophil undergoes a burst of respiratory activity at the site of the developing phagosome. There is increased activity of the hexose monophosphate shunt and oxygen is consumed. The superoxide anion is formed and it undergoes reduction to produce hydrogen peroxide. Further chemical reactions generate more toxic radicals such as the hydroxyl radical and singlet oxygen (Spitznagel, 1983; Beaman & Beaman, 1984). Neutrophil cytoplasmic granules fuse with the developing phagosome and

fusion continues after the formation of the phagosome is complete (Hirsch, 1962; Zucker-Franklin & Hirsch, 1964). The degranulation of the secondary granules followed by the primary granules, which was observed in rabbit neutrophils (Bainton, 1973), was not as apparent in human neutrophils (Pryzwansky et al., 1979).

Fusion of the primary granules with the phagosome releases myeloperoxidase into the phagosome lumen. In the presence of myeloperoxidase, hydrogen peroxide and halide ions cause the halogenation of bacterial proteins and further damage to bacterial components (Root & Cohen, 1981; Beaman & Beaman, 1984). Neutrophils also possess oxygen-independent bactericidal mechanisms. The primary granule components of human neutrophils which contribute to oxygen-independent killing are acid hydrolases, neutral proteases and cationic proteins. The role of the secondary granule components in the killing of intra-phagosomal bacteria is unclear (Rest et al., 1978; Wang-Iverson et al., 1978). Bacterial species differ in their susceptibility to the oxygen-independent and oxygen-dependent killing mechanisms of neutrophils (Mandell, 1974; Weiss et al., 1982; Vel et al., 1984; Casey et al., 1985; Bortolussi et al., 1987). Furthermore, the effectiveness of oxygen-dependent killing mechanisms may be reduced by the competition for oxygen which occurs between some bacteria and neutrophils in the presence of serum (Britigan & Cohen, 1986).

As noted above, some pathogenic bacteria avoid the toxic environment of the neutrophil's phagosomes by either escaping from the phagosome (Rikihisa & Ito, 1982) or inhibiting phagosome-granule fusion (Riley & Robertson, 1984; Bertram et al., 1986) within the neutrophil. The escape of Rickettsia tsutsugamushi from phagosomes into the cytoplasm of guinea pig neutrophils occurs before degranulation (Rikihisa & Ito, 1982). Riley and Robertson (1984) found there was minimal

degranulation of primary and secondary granules within bovine neutrophils after the phagocytosis of smooth strains of Brucella abortus. A nucleotide-like extract from a smooth strain of B. abortus preferentially inhibited the fusion of primary granules with phagosomes containing zymosan particles (Bertram et al., 1986). There was a slight inhibition of secondary granule degranulation.

The inhibition of phagosome-lysosome fusion was first observed in the macrophage. Lysosomes of cultured murine peritoneal macrophages showed infrequent signs of fusion with phagosomes containing intact Mycobacterium tuberculosis (Armstrong & Hart, 1971). A protein extract of M. tuberculosis increased the pH of murine macrophage lysosomes and it is suggested that this alkalinization may affect phagosome-lysosome fusion (Chicurel et al., 1988). Damaged or intact viable Mycobacterium avium (Frehel et al., 1986) and viable Mycobacterium leprae (Frehel & Rastogi, 1987; Sibley et al., 1987) also inhibited phagosome-lysosome fusion in cultured murine peritoneal macrophages. Frehel and Rastogi (1987) found that there was more fusion of lysosomes with phagosomes containing M. leprae if the bacteria had been treated with antibodies. Sibley et al. (1987) demonstrated increased phagosome-lysosome fusion in activated macrophages. Legionella pneumophila inhibited phagosome-lysosome fusion in human monocytes, but this was partially reversed by either treating the bacteria with antibodies or activation of the monocytes (Horwitz, 1983). L. pneumophila also inhibited acidification of its phagosome in monocytes (Horwitz & Maxfield, 1984). Brucella suis induced significantly less phagosome-lysosome fusion in murine peritoneal macrophages than did Micrococcus lysodeikticus (Oberti et al., 1981). Ryter and de Chastellier (1983) state that the inhibition of phagosome-lysosome fusion within phagocytes is never complete whatever the pathogen.

Elsbach (1980) observed that digestion and complete degradation of phagocytosed bacteria does not automatically follow phagosome-granule fusion. However, electron microscopy studies of phagocytosed bacteria *in vitro* or *in vivo* provide information on the ultrastructural damage that can occur to gram-positive and gram-negative organisms within the phagosomes of neutrophils. Ultrastructural features of digestion common to group A streptococci (Ayoub & White, 1969), non-encapsulated *E. coli* (Rozenberg-Arska *et al.*, 1985), *Staphylococcus epidermidis* (De Voe *et al.*, 1973b) and *B. abortus* (Young *et al.*, 1985) were reduced electron density and vacuolation of the bacterial cytoplasm, changes in cell wall appearance and disintegration of the cytoplasmic membrane. The rate of bacterial degradation is influenced by the species of bacteria, the type of phagocyte and possibly by the animal source of the phagocyte.

Biochemical methods using bacteria with radioactively-labelled components have enabled the identification of the macromolecules under attack within the neutrophil's phagosomes (Elsbach, 1980). For example, Thore *et al.* (1985) compared the degradation of a phagocytosed radio-labelled encapsulated strain of *Streptococcus pneumoniae* in the presence and absence of oxygen. The degradation of teichoic acid, RNA and DNA was similar in aerobic and anaerobic conditions, whereas the digestion of arachidonic acid was only apparent aerobically. Thus, the authors concluded that peroxidation of membrane lipids may be responsible for the fast aerobic killing of the organism by human neutrophils.

Bassøe and Bjerknes (1985) studied the intra-phagosomal degradation of seven species of bacteria by flow cytometry. The rates of degradation varied but bacterial proteins were more rapidly degraded than was the DNA of all species tested. An intra-phagosomal pH of between 5.2 and 5.4 was necessary for maximal bacterial degradation by the human leukocytes.

1.5 PHAGOCYTOSIS OF BACTEROIDES SPECIES

1.5.1 Chemotaxis

The culture filtrates and outer membranes of strains of B. fragilis, B. vulgatus and B. thetaiotaomicron induced some chemotaxis of rabbit neutrophils in a Boyden chamber (Adamu & Sperry, 1981). Subsequently, Sperry and Burns (1987) found the peptidoglycan of B. fragilis was chemotactic for rabbit neutrophils, whereas lysozyme-treated peptidoglycan was not chemotactic. Namavar et al. (1984) also demonstrated that culture filtrates of B. fragilis were moderately chemotactic for human neutrophils. The culture filtrates of Bacteroides gingivalis, B. asaccharolyticus and Bacteroides loeschei were not chemotactic. However, the culture filtrates of all Bacteroides species tested inhibited the chemotactic response of neutrophils to the stronger chemotactic factors of P. mirabilis. The culture filtrate and outer membrane of a B. fragilis strain were also able to inhibit the chemotactic response of neutrophils to E. coli (Namavar et al., 1987). LPS of this B. fragilis strain was chemotactic. Facultative bacteria such as S. aureus and Klebsiella pneumoniae also inhibited the chemotaxis of neutrophils towards E. coli. These groups (Namavar et al., 1987; Sperry & Burns, 1987) suggest that the ability of some B. fragilis components to inhibit neutrophil chemotaxis may enable the organism to establish an infection in association with facultative anaerobes before an influx of neutrophils occurs.

1.5.2 Opsonization and Phagocytic Killing

Activation of the alternative complement pathway in human NS facilitates the opsonization of B. fragilis and B. thetaiotaomicron (Bjornson & Bjornson, 1978; Tofte et al., 1980; Joiner et al., 1981), B. vulgatus (Tofte et al., 1980; Joiner et al., 1981), B. distasonis and B. ovatus (Joiner et al., 1981) and B. melaninogenicus (Tofte et al., 1980). The opsonization and phagocytic killing of B. fragilis and B. thetaiotaomicron required the alternative complement pathway and IgM (Bjornson et al., 1980). However, Tofte et al. (1980) showed that immunoglobulin and the classical complement pathway were necessary for optimal opsonization.

Activation of the alternative complement pathway in murine NS was necessary for the opsonization of B. fragilis and E. coli and their killing by murine neutrophils (Finlay-Jones et al., unpublished). Ellis and Barrett (1982) found that either murine NS, murine IS or both types of sera effectively opsonized B. fragilis.

Recently, Bjornson et al (1987) confirmed that the activation of the alternative complement pathway by B. fragilis and B. thetaiotaomicron fixed C3 to the bacterial surfaces in the form of C3bi and this did not require other serum components. However, the processes of attachment to neutrophil receptors, ingestion and intracellular killing of the bacteria did require other serum factors. IgM was the additional serum factor required for the opsonophagocytic killing of B. thetaiotaomicron but the additional serum factor(s) present in EGTA-treated pooled human NS and required by B. fragilis is unknown. Phagocytic killing of strains of B. fragilis and B. thetaiotaomicron used by Bjornson and Bjornson (1978) occurred in anaerobic conditions.

1.5.3 Killing by Abscess Neutrophils

Murine neutrophils from abscesses induced by the IP inoculation of *B. fragilis*, *E. coli* plus bran were unable to significantly kill *in vitro*, in the presence of NS, the bacteria they had phagocytosed *in vivo* (Hart *et al.*, 1986b). The abscess neutrophils were able to kill extra bacteria added *in vitro*. In contrast, abscess neutrophils containing *S. aureus* phagocytosed *in vivo* were able to kill the ingested bacteria *in vitro*. *S. aureus* were also rapidly killed within murine IA abscesses (Hart, unpublished observation).

Abscess neutrophils were less efficient at phagocytosing and killing bacteria *in vitro* than were neutrophils from the peripheral blood and induced peritoneal exudates of abscess-bearing mice (Hart *et al.*, 1986b). Extracellular NS enhanced the intracellular killing of bacteria via receptors on the surface of the murine neutrophils (Hart *et al.*, 1986a). Abscess neutrophils required extracellular NS for bactericidal activity and these neutrophils had increased expression of C3 receptors and similar expression of Fc receptors when compared to peripheral blood neutrophils. Bacteria released from abscess neutrophils by lysis of the phagocytes were susceptible to killing by peritoneal exudate neutrophils (Hart *et al.*, unpublished).

Thus the inability of abscess neutrophils to kill *in vitro* gram-negative bacteria, phagocytosed *in vivo*, is not due to the malfunctioning of the entire neutrophil population or to the resistance of some bacteria to intracellular killing, but to some, as yet unidentified, mechanism.

1.5.4 Inhibition of Phagocytic Killing of Facultative Anaerobes

Studies demonstrating the impairment of the phagocytosis and killing of facultative anaerobes by human neutrophils in the presence of Bacteroides species are summarized in Table 1.2. Connolly et al. (1984) found that the capsular polysaccharide of B. fragilis was responsible for the inhibition of phagocytic killing, whereas Jones and Gemmell (1986) demonstrated a role for B. fragilis LPS in this process. However, some studies have indicated that the ability to inhibit phagocytic killing of facultative anaerobes is not restricted to Bacteroides species (Dijkmans et al., 1985; Vel et al., 1985).

The concentration of the strain of Bacteroides species influences its ability to inhibit the phagocytic killing of facultative anaerobes. A concentration of more than 10^7 B. fragilis per millilitre was necessary before phagocytic killing of P. mirabilis was reduced (Ingham et al., 1981; Connolly et al., 1984). The pre-treatment of human serum with more than 10^5 Bacteroides per millilitre was necessary before there was impairment of the ability of the serum to opsonize P. mirabilis (Jones & Gemmell, 1982).

The ability of Bacteroides species to consume complement and therefore deprive the facultative anaerobe of opsonins is a frequently suggested mechanism for the impairment of neutrophil activity (Table 1.2). Vel et al. (1985) observed that in earlier studies where competition for opsonins was reported, it was often not stated if the anaerobes were subsequently killed. Vel et al. (1986) compared the interactions of B. fragilis and B. vulgatus with human neutrophils. Strains of both B. fragilis and B. vulgatus were able to inhibit the phagocytic killing of E. coli. This

Table 1.2: Effect of Anaerobes on the Phagocytosis and Killing of Facultative Anaerobes

Anaerobe	Facultative Anaerobe	Stage of Phagocytosis Inhibited	Mechanism of Inhibition	Reference
B. fragilis, B. melaninogenicus	E. coli	Ingestion	Competition for opsonins	Tofte et al. (1980)
B. fragilis	P. mirabilis	Phagocytic killing	Effect of anaerobe on serum	Ingham et al. (1981)
B. asaccharolyticus	P. mirabilis, E. coli, Klebsiella atlantae, Streptococcus faecalis	Phagocytic killing	Effect of anaerobe on serum	Ingham et al. (1981)
B. melaninogenicus,	P. mirabilis	Ingestion & phagocytic killing	Competition for opsonins	Jones & Gemmell (1982)
B. fragilis	" "	Ingestion	Competition for opsonins	
B. gingivalis	P. mirabilis	Phagocytic killing	Complement inactivation & inhibition of neutrophils	Namavar et al. (1983)
B. fragilis, B. asaccharolyticus, B. melaninogenicus	P. mirabilis	Phagocytic killing	-	Namavar et al. (1983)
Encapsulated B. fragilis, purified capsule ± B. fragilis	P. mirabilis	Phagocytic killing	Mediated by capsule	Connolly et al. (1984)
Non-encapsulated (?) B. ovatus	P. mirabilis	Phagocytic killing	?	Connolly et al. (1984)
B. fragilis	E. coli	Ingestion & intracellular killing	Competition for opsonins & limitations to neutrophil killing ability	Dijkmans et al. (1985)
B. fragilis	E. coli	Phagocytic killing	Competition for opsonins	Vel et al. (1985)
B. asaccharolyticus, B. fragilis lipopolysaccharide	gram-positives + gram-negatives	Ingestion	Reduced serum opsonic activity	Jones & Gemmell (1986)
B. fragilis, B. vulgatus	E. coli	Phagocytic killing	Competition for opsonins	Vel et al. (1986)

was related to their ability to consume complement. Although complement consumption resulted in the deposition of C3 on the surfaces of the anaerobes, it was not related to their susceptibility to phagocytic killing.

1.5.5 Effect of Bacterial Capsules

The role of the alternative or classical complement pathways in the opsonization of B. fragilis was not influenced by the presence or absence of a bacterial capsule (Tofte et al., 1980; Bjornson et al., 1983). However, the arrangement and extent of capsular material may affect opsonization (Bjornson et al., 1983).

An influence of the capsule on killing of B. fragilis by human neutrophils has been reported. More encapsulated animal-passaged B. fragilis survived phagocytosis and killing by neutrophils in human NS than did laboratory-passaged B. fragilis which possessed less capsular material (Simon et al., 1982). The ratio of bacteria to neutrophils was 1:2. Reid and Patrick (1984) were able to demonstrate reduced phagocytic killing of encapsulated B. fragilis in human NS only at a ratio of ten bacteria per neutrophil. Electron microscopy revealed few encapsulated B. fragilis in the phagosomes of neutrophils at this ratio, whereas intraphagosomal non-encapsulated B. fragilis were plentiful.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMALS

Male and female C3H/He (Inbr (N) 160) mice were supplied by the Small Animal Production Unit (SAPU), Massey University. The mice were provided with food and water ad libitum. One rabbit, which was used to raise immune serum (IS) to Bacteroides fragilis MFN 1110, was also supplied by SAPU. The animals were treated in accord with the code of ethical conduct set by the Animal Ethics Committee of Massey University.

2.2 MEDIA

Schaedler broth, brain heart infusion broth (BHIB) and Wilkins Chalgren (WC) agar were prepared from powders supplied by Difco Laboratories (Detroit, Michigan, USA). Sometimes WC agar was supplemented with 100 μg/ml Neomycin sulphate (Sigma, St Louis, Missouri, USA) and 7.5 μg/ml Vancomycin hydrochloride (Eli Lilly & Co., West Ryde, New South Wales, Australia). Phosphate-buffered saline (PBS) was adjusted to the osmolarity of mouse serum (333 mOsm/kg H_2O) as described by Sheridan and Finlay-Jones (1977). Foetal calf serum (FCS) was obtained from Gibco Laboratories (Madison, Wisconsin, USA) and RPMI 1640 medium was prepared from powder supplied by Flow Laboratories (Irvine, Scotland). The RPMI 1640 medium was buffered with a final concentration of 20 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid (HEPES; Sigma).

2.3 BACTERIA

Bacteroides fragilis MFN 1110, Bacteroides vulgatus MFN 15 and Escherichia coli MFN 60 were supplied by Dr M F Nulsen, Dept of Microbiology and Genetics, Massey University, Palmerston North, New Zealand. The bacteria had been isolated from abscesses induced in Balb/c mice by the IP inoculation of colonic and caecal contents from meat-fed mice (Nulsen et al., 1983). B. fragilis ATCC 23745 was obtained from the American Type Culture Collection (Rockville, Maryland, USA). The identities of the Bacteroides strains were confirmed by API An-Ident strips (Analytab Products, New York, USA) and the identity of E. coli MFN 60 by the API 20E system (Analytab Products).

The bacteria were sub-cultured no more than three times before being freeze-dried and stored at 0-4°C. In order to obtain stocks of bacteria for more immediate use, a freeze-dried ampoule was opened and the bacteria were cultured on WC agar. E. coli was grown for 24 hrs at 37°C in an aerobic environment. The Bacteroides strains were grown for 48 hrs at 37°C in an anaerobic chamber (Model SJ-3; Kaltec Pty Ltd, Edwardstown, South Australia) filled with an atmosphere of 10% H_2, 5% CO_2 and 85% N_2. The pO_2 of pre-reduced tap water in the anaerobic chamber was 13 mm Hg. Isolated colonies from the WC agar plates were suspended in 2 ml Schaedler broth, which was then diluted 10-fold. 100 μl was spread on to each of several WC agar plates and the plates were incubated under the appropriate atmospheric conditions. The bacteria were then scraped into Schaedler broth. They were snap frozen in small aliquots in liquid N_2 and stored at -70°C. Viable counts were performed after thawing and the concentrations of the bacteria were in the range of 0.5-6.0 x 10^{10} cfu/ml. A Petroff-Hauser bacteria counter (C A Hauser & Son, Philadelphia, Pennsylvania, USA) was used to obtain total counts of the thawed bacteria. The viable number of bacteria represented 80-100% of the total number of bacteria.

Killed bacteria were prepared by autoclaving (121°C, 15 min) suspensions of the bacteria in Schaedler broth. The bacteria were washed three times (955 g, 20 min, RT) in RPMI 1640 medium before total counts were made and the sterility of the suspension confirmed.

2.4 CAPSULES

Transmission electron microscopy (EM) was used to assess the encapsulation of thawed stocks of bacteria which had been incubated in NS and IS for 30 min at 37°C. Antibodies have been shown to stabilize capsules of Bacteroides species for the subsequent EM processing (Lambe et al., 1984). The bacteria were then processed for EM examination using a standard procedure (Section 2.14.1), except ruthenium red (Sigma) was added to the fixative and sodium cacodylate buffer to give a final concentration of 0.15%. Encapsulation of B. fragilis MFN 1110 was also assessed by periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining (Section 2.14.3) as did Strohm et al. (1983).

2.5 BRAN

The potentiating agent, bran, was prepared by the method of Nulsen (1982). 10 g of pure wheat bran (ProVita Kitchens, Flemington, Victoria, Australia) were ground to a fine suspension in 100 ml distilled water. The suspension was filtered through two layers of surgical gauze. After autoclaving (121°C, 15 min) the suspension was dispensed in 0.5 ml and 1.0 ml aliquots and stored at -20°C. The dry weight of the bran after dispensing was 41 mg/ml.

2.6 ABSCESS-INDUCING MIXTURES

Thawed stocks of B. fragilis MFN 1110, E. coli MFN 60 and bran were used in the preparation of the usual abscess-inducing mixture (AIM). The bacteria and bran were diluted in RPMI 1640 medium such that each mouse received 5 x 10^8 cfu of B. fragilis MFN 1110, 1 x 10^6 cfu of E. coli MFN 60 and 1 mg of bran when 0.05 ml of AIM was inoculated IP. Other abscess-inducing mixtures described in the results sections were prepared in a similar way.

2.7 EVALUATION OF ABSCESSES

The abscesses were dissected from the peritoneal cavities of mice killed by cervical dislocation. After the abscesses were weighed, they were placed in either 1 ml of saline for viable counts, 2.5 ml of 4% buffered formol saline for histological section, or Karnovsky's fixative (Appendix) for EM processing. Viable counts were determined by grinding the abscesses to even suspensions in scintered glass grinders, serially diluting the suspensions and placing 100 µl aliquots on WC agar with and without antibiotics. Fixed abscesses for histological studies were wax-embedded, sectioned and stained with haematoxylin-eosin. Gomori's one-step trichrome method was used to stain collagen (Luna, 1968). Some wax-embedded abscesses were reprocessed for EM (Section 2.14.4).

An indirect fluorescent antibody test was used to detect bacterial antigens within abscesses. Histological sections of abscesses were dewaxed by immersion in xylol for 2 min, followed by absolute ethanol and 70% ethanol for 30 s each and then rinsed in distilled water. The dewaxed sections were then treated with trypsin to reduce background fluorescence (Mera et al., 1980). They were rinsed in TRIS-saline buffer, pH 7.8 and incubated in 0.05% trypsin (Serva, Heidelberg, West Germany) in

TRIS-saline buffer, pH 7.8, plus 0.1% $CaCl_2$ at 37°C for 40 min. After being left in TRIS-saline buffer, pH 7.8 at 4°C overnight, they were rinsed twice in PBS. The dewaxed, trypsinized sections were then stained with anti-B. fragilis MFN 1110 or anti-E. coli MFN 60 sera as described in Section 2.9.

2.8 SERA

Fresh serum was collected from normal mice (NS) that were anaesthetized and bled from the retro-orbital venous plexus. Pooled blood was left to clot at room temperature for about 15 min, ringed and placed on ice for 1-2 hrs. Approximately 1.5 g of Sera Sieve (Hughes & Hughes Ltd, Essex, England) was layered on top and the blood centrifuged (955 g, 30 min, RT). Serum was collected, filtered through 0.22 μ membrane filters and used immediately. NS did not have detectable antibodies to Bacteroides strains used in this study, but undiluted NS gave a positive result when tested with E. coli MFN 60 in an indirect fluorescent antibody test. Heat-inactivated serum (HNS) was prepared by incubating NS at 56°C for 30 min. HNS was used immediately or stored at -20°C.

IS were obtained from mice immunized SC with 0.1 ml of PBS containing viable, washed bacteria. The inoculum per mouse was either 0.5 - 1.0 x 10^9 cfu of Bacteroides strains or 1.0 x 10^7 cfu of E. coli MFN 60 given three times per week for two weeks. A further inoculation was given in the fourth week, and the mice were bled one week later. All IS were heat-inactivated and stored in aliquots at -70°C. The titres of thawed sera were determined by an indirect fluorescent antibody test (Section 2.9). Anti-B. fragilis MFN 1110 sera had titres ranging from 1/128 to 1/512. The titre of anti-B. fragilis ATCC 23745 serum was 1/256. Anti-B. vulgatus MFN 15 serum had a titre of 1/128. The titres of anti-E. coli MFN 60 sera ranged from 1/16 to 1/64.

A rabbit was immunized SC with 1 ml of 5 x 10^9 cfu/ml of B. fragilis MFN 1110 in saline using the above immunization protocol, except 5 x 10^9 heat-killed bacteria mixed with an equal volume of Freund's incomplete adjuvant were used for the first inoculation. The titre of the IS, as assessed by indirect fluorescent antibody staining, was 1/256.

2.9 INDIRECT FLUORESCENT ANTIBODY TEST

An indirect fluorescent antibody test was used to titrate sera. Thawed washed suspensions of bacteria were diluted to contain 10^8 cfu/ml. 10 μl aliquots were placed on alcohol-washed glass microscope slides and allowed to air-dry. These were heat- or acetone-fixed and stored at -20°C. 10-20 μl of serial 2-fold dilutions of the thawed sera were placed on the appropriate slides. Negative and positive controls were included in each test. The slides were incubated for 30 min at 37°C, washed for 30 min in two rinses of PBS and one of distilled water, and air-dried. 10-20 μl aliquots of fluorescein-conjugated sheep IS to mouse immunoglobulin (diluted 1/15; Amersham International, Buckinghamshire, England) or fluorescein-conjugated sheep IS to rabbit immunoglobulin (diluted 1/30; Wellcome Reagents Ltd, Beckenham, England) were added to the slides and the slides were incubated and washed as before. The slides were mounted in glycerol: 0.5 M carbonate buffer, pH 9.5 (9 parts:1 part, final pH 8.5; Kawamura, 1977). Slides were examined for fluorescence under oil immersion (magnification x 1250) with a Leitz Ortholux II microscope fitted with a Ploemopak 2.2 fluorescence illuminator and an HBO-200 W mercury arc burner. An I2 filter block (BP 450-490 nm excitation filter and LP 515 nm suppression filter) was used.

B. fragilis MFN 1110 antigens in histological sections of abscesses were detected by indirect fluorescent antibody staining as above, using rabbit anti-B. fragilis serum diluted ½

and absorbed with acetone-dried mouse liver powder (Appendix) to remove rabbit antibodies to mouse antigens. The secondary antibody was sheep anti-rabbit immunoglobulin. E. coli MFN 60 antigens were identified by mouse anti-E. coli serum, diluted ½, and fluorescein-conjugated goat anti-mouse immunoglobulin (diluted 1/40; Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA).

2.10 OPSONIZATION IN VITRO

E. coli MFN 60, B. fragilis MFN 1110 and B. vulgatus MFN 15 were opsonized separately. Thawed stocks of bacteria, NS or NS plus IS were mixed with RPMI 1640 medium to give 2 x 10^6 or 2 x 10^9 cfu/ml in 10% serum. Thawed stocks of bran and NS were mixed with RPMI 1640 medium to give 4 mg/ml of bran in 10% NS. The bacteria or bran were incubated in a shaking waterbath (Heto Laboratory Equipment, Denmark) at 37°C for 30 min, washed twice (955 g, 20 min, RT) and resuspended in RPMI 1640 medium to the appropriate concentration. The opsonized bacteria or bran were used immediately.

2.11 IN VITRO PHAGOCYTIC KILLING ASSAY

The assay of in vitro phagocytic killing was based on the method of Finlay-Jones et al. (1984). Usually the assay mixture consisted of 5 x 10^6 peritoneal leukocytes, 5 x 10^5 or 5 x 10^8 cfu of bacteria and 10% serum in a final volume of 1 ml of RPMI 1640 medium. Experiments were carried out in 8 ml glass Kimax tubes or 1.5 ml polypropylene Eppendorf microcentrifuge tubes which were mixed at 20 rpm on a rotating platform (Nutator; Clay Adams, Parsippany, New Jersey, USA) in a 37°C incubator. In experiments involving anaerobic assays, mixtures were prepared and incubated in an anaerobic chamber using pre-reduced media. In experiments involving joint aerobic and anaerobic assays, all

mixtures were prepared in the anaerobic chamber and those for aerobic incubation were then removed from the chamber and aspirated 5-10 times with a Pasteur pipette. These aerobic assay mixtures were incubated at 37°C on a shaker (G10 Gyrotory shaker; New Brunswick Scientific Co Inc., New Brunswick, New Jersey, USA) at 220 rpm. Samples of 100 µl were removed at 0, 60 and 120 min, and added to 0.9 ml or 9.9 ml of saline. Leukocytes were disrupted by 15 s of sonication at a setting of 4 µ (Soniprep 150 Ultrasonic Disintegrator; MSE Scientific Instruments, Crawley, Sussex, England) and viable counts performed. In some cases, the leukocyte morphology and the leukocyte-associated bacteria in samples were examined using cytocentrifuge-prepared smears (8.1 g, 5 min; Shandon Cytospin, Cheshire, England) stained with Giemsa stain (BDH Chemicals Ltd, Poole, England).

Peritoneal leukocytes were obtained 3.5 hrs after IP inoculation of mice with 1 ml BHIB prepared with tap water. After cervical dislocation, mice were inoculated IP with up to 5 ml PBS, the abdomen massaged for 1 minute, the skin resected and fluid withdrawn from the peritoneal cavity. The leukocytes were washed three times (106 g, 5 min, RT), under aerobic or anaerobic conditions, in PBS with a 1 ml FCS underlay. Finally the leukocytes were suspended in RPMI 1640 medium at a concentration of 2 x 10^7/ml. Apart from the centrifugations, the leukocytes were kept on ice during all the preparation stages. Peritoneal leukocytes obtained by this method were 52.5 ± 8.7% neutrophils, 21.2 ± 8.5%, macrophages, 24.5 ± 11.0% lymphocytes and monocytes and 1.8 ± 1.5% basophils and eosinophils.

Thawed stocks of bacteria diluted in RPMI 1640 medium immediately before use were most frequently used in the assays. Sometimes the bacteria were opsonized (Section 2.10) before inclusion in the assay mixtures.

Results were usually expressed as the change in the viable count of bacteria (ΔB) at time, t. This was calculated using the following formula:

$$\Delta B = \log_{10} (\text{cfu/ml bacteria})_t - \log_{10} (\text{cfu/ml bacteria})_0$$

2.12 IN VITRO INTRACELLULAR KILLING ASSAY

The assay of intracellular killing was based on the methods of van Furth et al. (1978), Leijh et al. (1980) and Dijkmans et al. (1985). Eppendorf microcentrifuge tubes containing suspensions of 5 x 10^6 peritoneal leukocytes and 5 x 10^8 cfu of opsonized bacteria in 1 ml of RPMI 1640 medium (sometimes with 1% NS) were incubated at 37°C on a rotating platform (Nutator; Clay Adams) for 3 min. Phagocytosis was stopped by shaking the tubes through ice. The extracellular bacteria were removed by washing the leukocytes three times (106 g, 5 min, RT) in ice-cold RPMI 1640 medium underlaid with FCS. The leukocytes were resuspended in 1 ml of 10% NS or RPMI 1640 medium and reincubated under rotation at 37°C for up to 120 min. After 0, 60 and 120 min, 100 µl samples were removed and added to 0.9 ml saline underlaying with FCS. The leukocytes were centrifuged, resuspended in 1 ml of saline and transferred to a ¼ oz bottle for sonication (Section 2.11). After the leukocytes were disrupted, viable counts were performed. Sometimes a separate 50 µl sample was taken for the preparation of cytocentrifuge smears (Section 2.11).

2.13 ENUMERATION OF BACTERIA AND LEUKOCYTES FROM THE MURINE PERITONEAL CAVITY

Peritoneal leukocytes were elicited and collected as described in Section 2.11, except leukocytes were collected in 2.5 ml PBS and the peritoneal contents from the mice were not pooled.

After three washes (106 g, 5 min, RT), underlaying with FCS, the peritoneal leukocytes were sonicated (Section 2.11) and the number of viable leukocyte-associated bacteria was determined from plate counts. Total leukocyte counts were performed using a haemocytometer and differential counts and counts of leukocyte-associated bacteria were performed on Giemsa stained cytocentrifuge-prepared smears.

2.14 TRANSMISSION ELECTRON MICROSCOPY

2.14.1 Standard Procedure

Suspensions of leukocytes were fixed in equivalent volumes of Karnovsky's fixative, pH 7.4 (Appendix) at 0-4°C. Suspensions were fixed for at least 1 hr. Approximately 1 ml aliquots were centrifuged in Eppendorf microcentrifuge tubes in a microcentrifuge (8800 g, 5 min, RT; Eppendorf, West Germany) (Malamed, 1963). The supernatants were discarded and the bases of the tubes containing the pellets were cut away from upper parts of the tubes. The pellets, still in the tube bases, were placed in 0.1 M sodium cacodylate buffer, pH 7.4 (Appendix) in glass vials and left overnight, or up to 1 mth at 0-4°C. Post-fixation of the pellets in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 (Appendix) was carried out for 2 hrs at room temperature. The pellets were then either rinsed three times in distilled water for a total of 15 min, or stained *en bloc* in 2% aqueous uranyl acetate for 1 hr at room temperature. The pellets were dehydrated in 30%, 50%, 70%, 90% and 95% acetone for a total of 50 min, followed by 3-4 immersions in 100% acetone, each lasting 30 min. The pellets were then removed from the tube bases if they had not already been dislodged during previous stages. A 1:1 mixture of 100% acetone and Spurr's resin (Polysciences Inc., Warrington, Pennsylvania, USA) was used to infiltrate the pellets, which were rotated (Taab Vari-

Speed Rotator; Taab Laboratories Equipment Ltd, Berkshire, England) for 60 min at room temperature, prior to leaving the pellets in Spurr's resin overnight. The pellets were infiltrated with fresh Spurr's resin for 30 min under rotation before transferral of the pellets to Spurr's resin in polythene 'Beem' capsules (Taab Laboratories Equipment Ltd). The samples were cured in an embedding oven (Taab Laboratories Equipment Ltd) at 70°C for approximately 24 hrs.

Suspensions of bacteria were processed for EM as above, except for one modification. After the fixed bacteria had been pelleted in microcentrifuge tubes and the supernatants discarded, the pellets were warmed to 45°C and one drop of molten 2% agar in tryptone medium (Appendix) was mixed with the pelleted bacteria and allowed to solidify. The bases of the tubes containing pellets were then cut away and the pellets processed for EM.

Abscesses were either diced or left intact with their outer surfaces slit with a scalpel blade to facilitate penetration of the fixative. The abscesses were then placed in fixative and processed for EM. Intact abscesses were cut into quarters at the end of the dehydration stage.

Once the blocks were cured 1 µm-thick sections were cut on an LKB-Huxley ultramicrotome (LKB-Produkter AB, Bromma, Sweden) using glass knives and examined by phase contrast microscopy. After trimming of the block to contain the area of interest ultra-thin (55-60 nm) sections were cut on a Reichert Ultracut E ultramicrotome (AO Reichert Scientific Instruments, Buffalo, New York, USA) using a diamond knife (Diatome Ltd, Bienne, Switzerland). The sections were collected on 200 or 300 mesh copper grids (Graticules Ltd, Tonbridge, England) and stained with uranyl acetate and lead citrate, or lead citrate alone if the samples had been stained en bloc in uranyl acetate (Appendix). A Philips EM

201C high resolution electron microscope was used to view the sections.

2.14.2 Peroxidase Cytochemistry

The primary granules and phagosomes of murine peritoneal neutrophils were labelled for peroxidase activity by the method of Bainton (1973). Leukocyte suspensions were fixed in an equal volume of 1.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 with 1% sucrose (Appendix) for at least 1 hr at 0-4°C. The leukocytes were then washed three times (106 g, 5 min, RT) in 0.1 M sodium cacodylate buffer, pH 7.4 with 7% sucrose and left overnight in the same buffer at 0-4°C. After centrifugation to remove the buffer, the leukocytes were resuspended in Graham's and Karnovsky's medium for peroxidase and left for 30 min at 25°C. The medium of Graham and Karnovsky (1966) was prepared by dissolving 5 mg of 3-3' diaminobenzidine tetrahydrochloride in 10 ml of 0.05 M TRIS-HCl buffer, pH 7.6 containing 0.01% H_2O_2 and 5% sucrose. The leukocytes were washed twice (106 g, 5 min, RT) in 0.05 M acetate-Veronal buffer, pH 7.4 with 7% sucrose (Appendix) and were pelleted during the third wash in the microcentrifuge. The pelleted leukocytes were post-fixed in 1% osmium tetroxide in acetate-Veronal buffer for 1 hr at room temperature. En bloc staining in 0.5% uranyl acetate in 0.05 M acetate-Veronal buffer, pH 7.4 with 7% sucrose was carried out for 1 hr at room temperature. The leukocytes were then dehydrated in acetones, infiltrated with and embedded in Spurr's resin (Section 2.14.1). Sectioning and staining of the sections was done as in Section 2.14.1. Negative controls involved incubating the leukocytes in Graham's and Karnovsky's medium, from which 3-3' diaminobenzidine tetrahydrochloride (Sigma) or hydrogen peroxide had been omitted.

2.14.3 Periodate-Thiocarbohydrazide-Silver Proteinate Staining

Periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining of murine peritoneal neutrophil secondary granules was evaluated by the method of Fittschen et al. (1983). The leukocytes were fixed, washed and stored overnight at 0-4°C as in Section 2.14.2. The leukocyte suspension was then split into three samples. The first sample was centrifuged (106 g, 5 min, RT) and the cells incubated for 3 hrs at 37°C in a solution of 275 units/ml of α-amylase from porcine pancreas (Sigma) in 0.09 M phosphate buffer. As a control, the second sample was incubated in 0.09 M phosphate buffer alone. The third sample had no α-amylase treatment. All samples were then pelleted in the microcentrifuge and dehydrated in acetones, infiltrated with and embedded in Spurr's resin (Section 2.14.1).

Sectioning was done as in Section 2.14.1, except sections were collected on stainless steel 200 mesh grids (Polysciences, Inc.). The sections were oxidized in 1% periodate (Serva) for 30 min, rinsed in distilled water, and treated for 40 min with 2% thiocarbohydrazide (Sigma) in 20% acetic acid. The sections were then rinsed rapidly in 10% acetic acid followed by distilled water. Next the sections were treated with 1% silver proteinate (Roques; BDH Chemicals Ltd) in the dark, followed by a rinse in distilled water. In control sections periodate, thiocarbohydrazide or both solutions were omitted from the staining procedure as recommended by Courtoy and Simar (1974).

2.14.4 Reprocessing of Paraffin Wax-Embedded Abscesses

The method used was based on that of Johannessen (1977). The paraffin wax blocks containing the abscesses were melted and the abscesses placed in two changes of xylol, followed by two changes of absolute ethanol. The abscesses were then placed in 95% ethanol, followed by 70% ethanol and water. Individual abscesses were placed, cut surface uppermost, under a stereo microscope alongside the appropriate haematoxylin-eosin stained section. Using the section as a guide, areas of interest were dissected from the abscess and washed twice in 0.1 M sodium cacodylate buffer, pH 7.4 for a total of 2 hrs. The abscesses were left in buffer overnight at 0-4°C. Post-fixation in 2% osmium tetroxide and the remaining EM processing were carried out as for Section 2.14.1.

2.15 STATISTICS

Two-sided unpaired t-tests were used for statistical analysis of data.

CHAPTER 3

A MODEL OF INTRA-ABDOMINAL ABSCESS DEVELOPMENT IN C3H MICE

3.1 INTRODUCTION

The development of IA abscesses in humans is influenced by the causative event (e.g. gross peritoneal soiling), the concentration and range of infecting bacterial species and the effectiveness of the host defence mechanisms (Haase & Harding, 1985). Although B. fragilis represents less than 1% of the colonic normal flora, it is more frequently present in these infections than B. vulgatus which is a major component of the colonic normal flora (Bartlett, 1981). Bacteria are removed from the peritoneal cavity by clearance through the diaphragmatic lymphatics, opsonization and phagocytic killing, and by trapping of the bacteria, either by fibrin or within adhesions between visceral surfaces (Dunn et al., 1985b). Murphy (1976) considers an abscess to be the result of the host's inability to eradicate the bacteria by phagocytosis alone. Infection of the peritoneal cavity evokes an acute inflammatory response characterized by an influx of neutrophils (Bartlett, 1981). However, opinions differ on the effectiveness of the neutrophils, as one of the defence mechanisms operational in the peritoneal cavity, to eradicate the bacteria (Dunn et al., 1985b; Freischlag et al., 1986; Skau et al., 1986). Since abscesses prevent the spread of the bacteria, they too can be considered a host defence, albeit an ineffective one. B. fragilis and E. coli persisted in IA abscesses in mice for up to ten weeks (Nulsen et al., 1983). Hart et al. (1986b) showed that murine neutrophils from IA abscesses induced by B. fragilis, E. coli and bran could not kill in vitro, in the presence of NS, the bacteria they had phagocytosed in vivo.

Several animal models have been used to study the pathogenesis of IA abscess formation (Onderdonk et al., 1977; Joiner et al., 1980b;, McConville et al., 1981; Shapiro et al., 1982; Nulsen et al., 1983; Patrick et al., 1984; Rotstein et al., 1985b; Verweij-van Vught et al., 1985; Thadepalli et al., 1986;

Verweij-van Vught et al., 1986). The virulence of B. fragilis ATCC 23745 in a rat model of IA sepsis has been attributed to the bacterial capsular polysaccharide (reviewed by Onderdonk et al., 1984). Verweij-van Vught et al. (1986) compared the virulence of B. fragilis with that of B. vulgatus in an SC model of abscess formation. The authors found that B. vulgatus was more rapidly cleared from SC sites in mice than was B. fragilis, although both organisms caused abscesses to develop when combined with E. coli.

A murine model of IA abscess formation (Nulsen et al., 1983) was used in the experiments described in this chapter to study the phagocytic response and abscess development in the peritoneal cavities of C3H mice following their IP inoculation with a variety of abscess-inducing mixtures. A combination of quantitative, histological and ultrastructural techniques was used to achieve the following aims:

(i) to demonstrate that the neutrophil is the dominant cell type in the murine model of IA abscesses and that bacteria persist in the abscesses in intra- and extra-cellular sites;

(ii) to compare the abscess-inducing ability of a murine strain of B. fragilis with that of B. fragilis ATCC 23745 and a murine strain of B. vulgatus;

(iii) to compare the infection with abscess-inducing mixtures containing either B. fragilis or B. vulgatus in terms of the phagocytic response within the peritoneal cavity and the fate of the bacteria within neutrophils.

3.2 RESULTS

3.2.1 Encapsulation of Bacteria

The possession of capsules by bacteria used in this study was assessed by transmission EM. Ruthenium red staining and PA-TCH-SP staining of *B. fragilis* showed the presence of capsules of varying densities (Fig.3.1a & b). *B. vulgatus* was also encapsulated (Fig.3.1e). Unpassaged *B. fragilis* ATCC 23745 appeared to lack a capsule (Fig.3.1c), whereas *B. fragilis* ATCC 23745 which had been passaged through murine IA abscesses five times developed capsules (Fig.3.1d). *E. coli* had a thin layer of capsular material (Fig.3.1f).

3.2.2 Abscess Development

Macroscopic IA abscesses developed in mice within three days of inoculation with an abscess-inducing mixture consisting of $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli* MFN 60 and 1 mg of bran (AIM). By day 6 92% of the mice had developed 1-6 abscesses per mouse (Table 3.1). After day 13 there were fewer abscesses per mouse, but after ten weeks 71% of the mice still had abscesses. A range of abscess weights was found throughout the ten weeks post-inoculation and during this period there was an increase in the weight of the abscesses per mouse (Table 3.1). By ten weeks there were 1-2 abscesses per mouse, with an abscess weight per mouse of 501 $\pm$ 265 mg.

B. fragilis and *E. coli* were cultured from IA abscesses during the ten weeks post-inoculation with AIM in numbers similar to those in the inoculum (Fig.3.2). After ten weeks there were $3.2 \pm 3.8 \times 10^8$ *B. fragilis* and $2.0 \pm 1.2 \times 10^6$ *E. coli* per abscesses per mouse, a ratio of 160 *B. fragilis*:1 *E. coli*.

Figure 3.1: Encapsulation of Bacteria

Electron micrographs of bacteria stained with ruthenium red (a, c-f) and by the PA-TCH-SP method (b) to detect capsules.
(a) & (b) *B. fragilis* MFN 1110, x 12021, (c) *B. fragilis* ATCC 23745, x 16657, (d) *B. fragilis* ATCC 23745 5x abscess-passaged, x 16657, (e) *B. vulgatus* MFN 15, x 16657 and (f) *E. coli* MFN 60, x 38186.

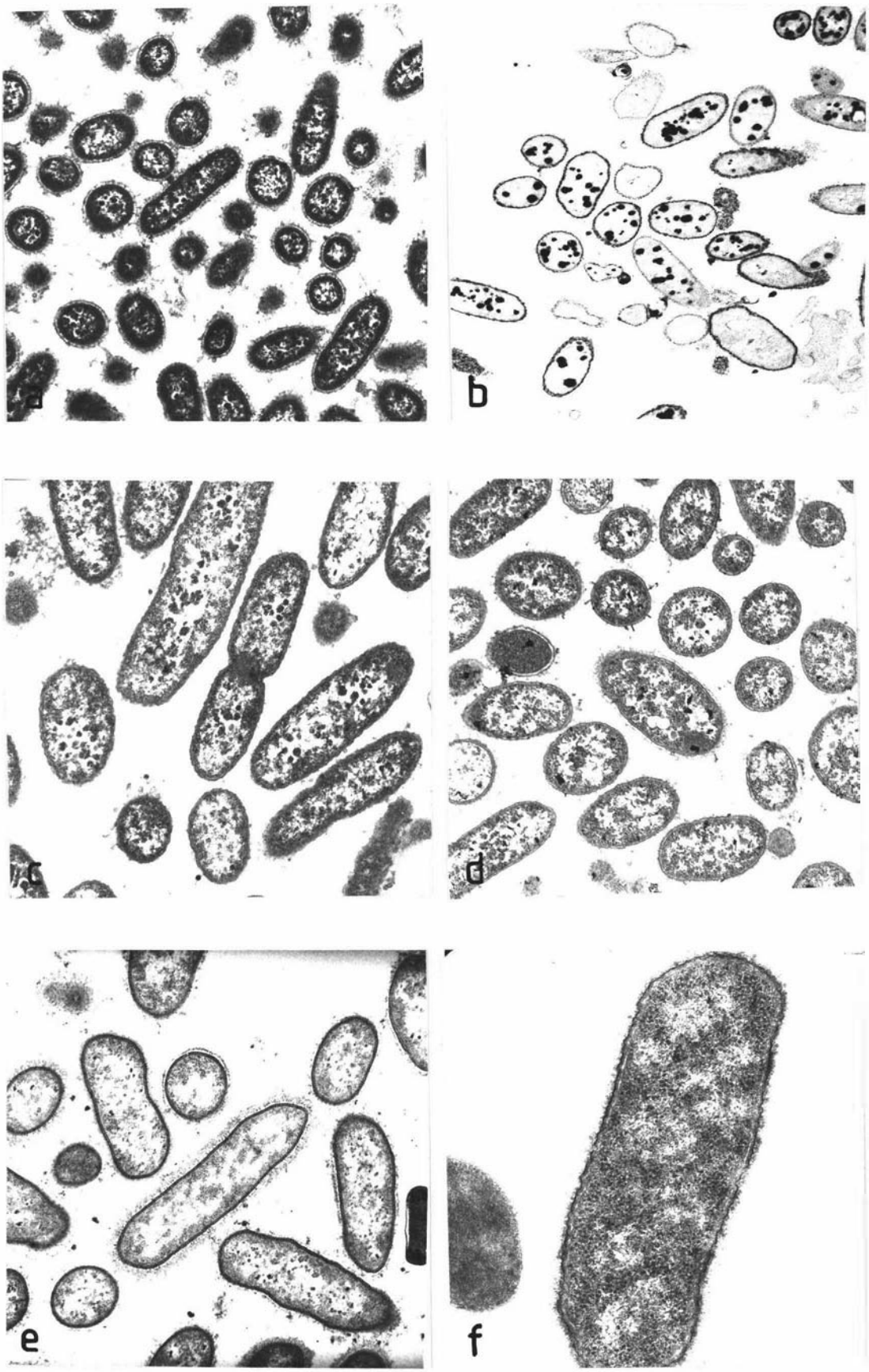

a
b
c
d
e
f

Table 3.1: Abscesses induced by *B. fragilis*, *E. coli* and bran[a]

Days post-inoculation	No. mice examined	Incidence (%)[b]	Range of abscess numbers/ mouse	Abscess weight (mg/mouse)[c] (Range of abscess weights in mg)
6	47	92	1-6	121 ± 64 (6 - 195)
13	21	90	1-5	169 ± 88 (7 - 179)
21	12	92	1-3	267 ± 143 (39 - 461)
28	7	71	1-2	96 ± 67 (24 - 168)
40	7	100	1-2	147 ± 114 (9 - 216)
70	7	71	1-2	501 ± 265 (171 - 487)

a Mice were inoculated IP with 0.05 ml of a mixture containing bran (1 mg/mouse), *B. fragilis* MFN 1110 ($5x10^8$ cfu/mouse) and *E. coli* ($1x10^6$ cfu/mouse)

b Incidence of abscesses > 5 mg

c Weight of abscesses > 5 mg, mean ± SD

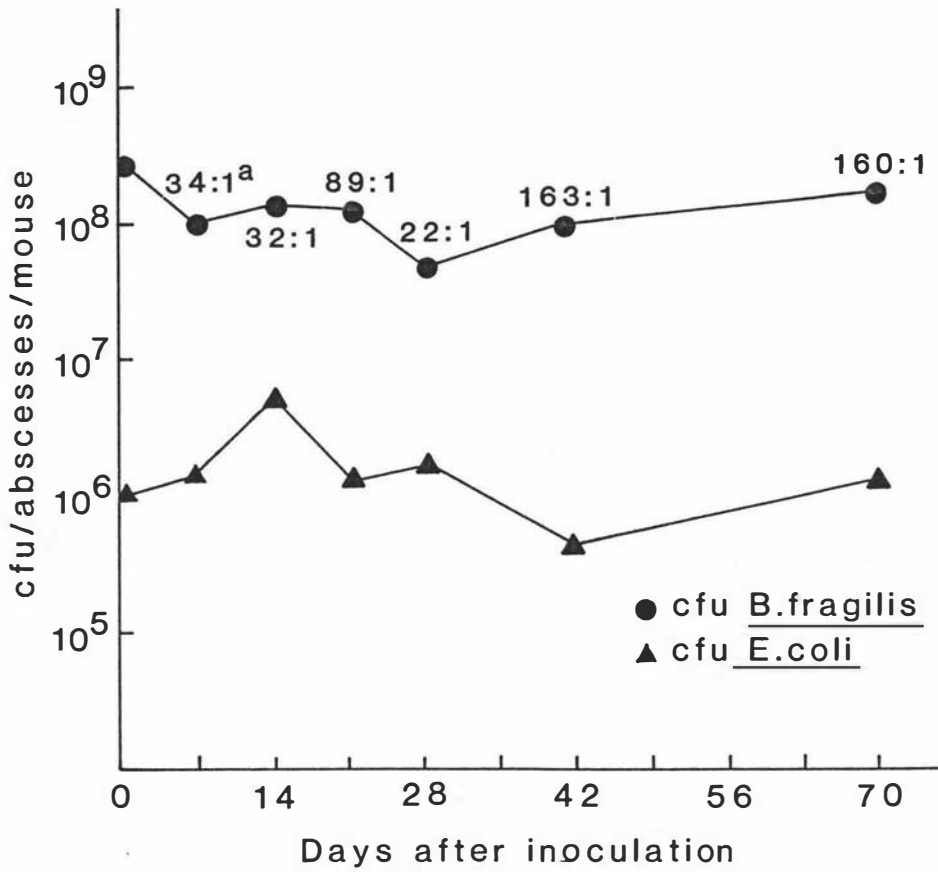


Figure 3.2: Viable bacteria in abscesses induced by B. fragilis, E. coli and bran. Mice were inoculated IP with 0.05 ml of a mixture containing bran (1 mg/mouse), B. fragilis MFN 1110 ($5x10^8$ cfu/mouse) and E. coli ($1x10^6$ cfu/mouse). The bacterial contents of the abscesses were determined. The day 0 value represents the bacterial inoculum. Abscesses from 3-5 mice were cultured on each of the days sampled, except day 21 (10 mice).

a. cfu B. fragilis:cfu E. coli

Abscesses were located at various sites in the peritoneal cavity, including the groin, mesentery, serosal surface of the intestines, peritoneum (not at the site of inoculation) and in the vicinity of the stomach. From day 40 onwards, some individual abscesses were found adhering to both the mesentery, small intestine and the peritoneum. In some instances these abscesses had burst through the peritoneum and were draining to the exterior of the mouse. The older abscesses were well vascularized.

When bran was omitted from AIM one abscess per mouse had developed in 50% of mice six days post-inoculation (Table 3.2). The abscesses were significantly smaller than those induced by AIM ($p < 0.05$) and less B. fragilis and E. coli were cultured from these abscesses at day 6 ($p < 0.05$, Fig.3.3). In the absence of bran, the ratio of B. fragilis to E. coli was 5:1 by day 6, mainly due to the greater decrease in the number of viable B. fragilis.

B. fragilis ATCC 23745 (passaged five times in murine IA abscesses), when combined with bran or bran and E. coli, induced abscesses to form which persisted for at least three weeks (Table 3.2, Fig.3.3). Six days after inoculation with B. fragilis ATCC 23745 alone, none of 12 mice examined had developed IA abscesses. A mixture of B. fragilis ATCC 23745, bran and E. coli caused 1-2 abscesses per mouse to develop in all mice examined six days post-inoculation. A similar situation was found on day 21. In the absence of E. coli, the IP inoculation of B. fragilis ATCC 23745 and bran resulted in a lower incidence but similar number of abscesses by day 6, and by day 21 60% of mice had one abscess each. Three weeks post-inoculation abscesses caused by B. fragilis ATCC 23745, E. coli and bran were not significantly heavier than those induced in the absence of E. coli and both types of abscesses contained similar numbers of B. fragilis (2-3 x

Table 3.2: Abscesses induced by Bacteroides species, E. coli and bran

Inoculum[a] (per mouse)	Days post-inoculation	No. mice examined	Incidence (%)[b]	Range of abscess numbers per mouse	Abscess weight (mg/mouse)[c] (Range of abscess weights in mg)
A. $5x10^8$ B. fragilis MFN 1110 $1x10^6$ E. coli	6	12	50	1	21 ± 19[e] (8 - 57)
B. $5x10^8$ B. fragilis ATCC 23745[d] $1x10^6$ E. coli 1 mg bran	6	7	100	1-2	58 ± 36[f] (6 - 100)
	21	5	100	1-3	111 ± 78 (19 - 139)
C. $5x10^8$ B. fragilis ATCC 23745[d] 1 mg bran	6	7	71	1-3	68 ± 51 (12 - 79)
	21	5	60	1	62 ± 8 (54 - 70)
D. $5x10^8$ B. vulgatus $1x10^6$ E. coli 1 mg bran	6	7	86	2	94 ± 27[g] (28 - 91)
	21	5	80	1	53 ± 49 (13 - 122)

a Mice were inoculated IP with 0.05 ml of mixtures A-D
b Incidence of abscesses > 5 mg
c Weight of abscesses > 5 mg, mean ± SD
d Passaged 5x in murine IA abscesses
e $P < 0.05$ v B. fragilis MFN 1110, E. coli and bran (Table 3.1)
f $P < 0.05$ v B. fragilis MFN 1110, E. coli and bran (Table 3.1)
g $P < 0.05$ v B. fragilis MFN 1110, E. coli and bran (Table 3.1)

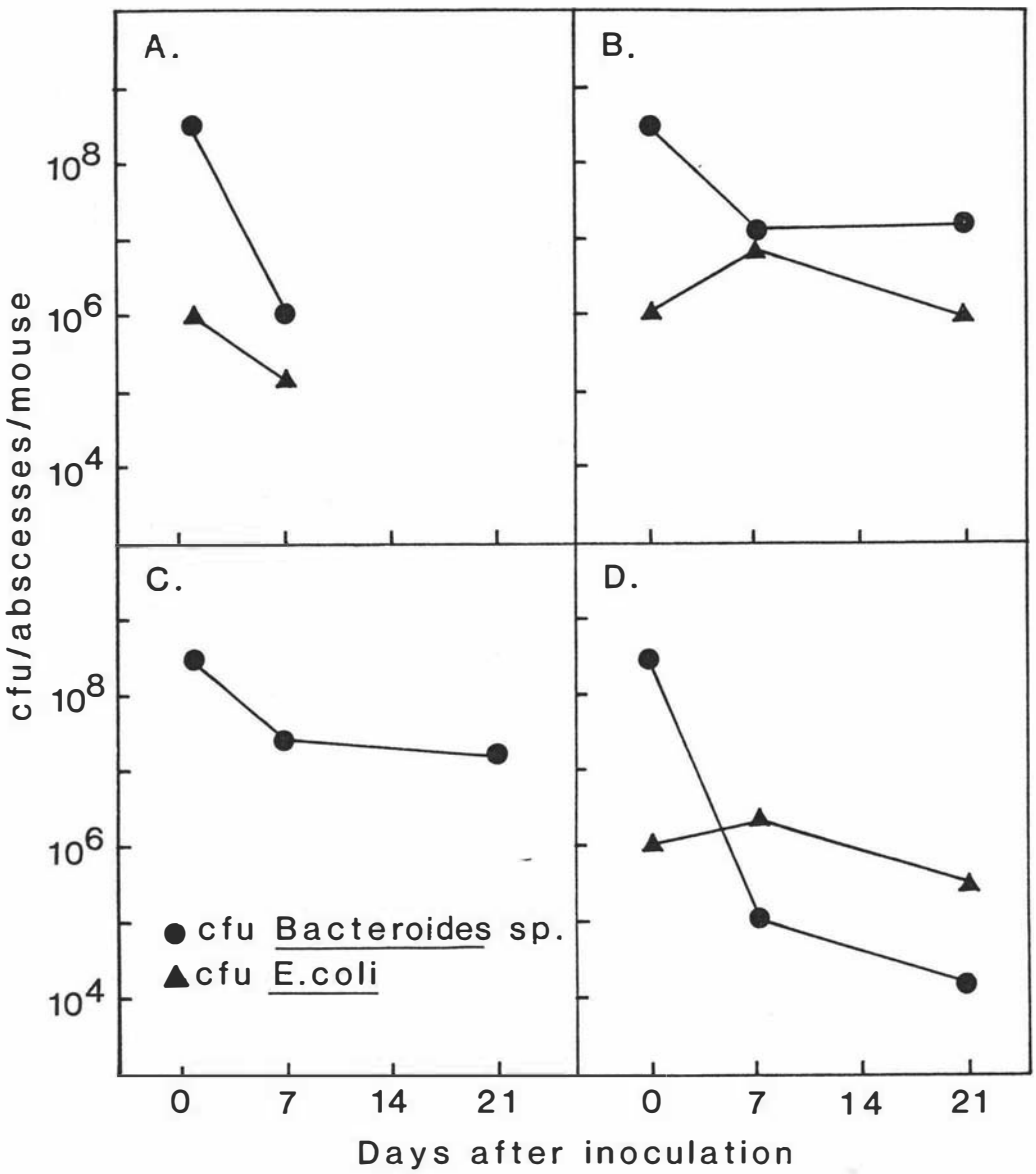


Figure 3.3: Viable bacteria in abscesses induced by Bacteroides species, E. coli and bran. Mice were inoculated IP with 0.05 ml of a mixture containing either B. fragilis MFN 1110 and E. coli (A), B. fragilis ATCC 23745[a], E. coli and bran (B), B. fragilis ATCC 23745[a] and bran (C) or B. vulgatus, E. coli and bran (D). The bacterial contents of the abscesses were determined. The day 0 value represents the bacterial inoculum. Abscesses from 3-5 mice were cultured on each of the days sampled.

[a] passaged 5x in murine IA abscesses

10^7 cfu/abscesses/mouse).

In comparison to abscesses induced by AIM, those induced by B. fragilis ATCC 23745, E. coli and bran were smaller on day six ($p < 0.05$; Table 3.2 cf. Table 3.1). Similar numbers of E. coli were cultured from both abscesses at days 6 and 21. On day 6 there were no significant differences between the numbers of B. fragilis ATCC 23745 and the numbers of B. fragilis MFN 1110 cultured from the two types of abscesses. However, by day 21 there were significantly fewer viable B. fragilis ATCC 23745 in abscesses induced by this organism, E. coli and bran, compared to viable B. fragilis MFN 1110 in abscesses induced by AIM ($p < 0.05$; Fig.3.3 cf. Fig.3.2).

On day 6 there was a lower incidence of abscesses following the IP inoculation of B. vulgatus, E. coli and bran when compared to AIM (Table 3.2 cf. Table 3.1). However, abscess-bearing mice were found on day 21. Abscesses induced by B. vulgatus, E. coli and bran were significantly smaller than their AIM counterparts on day 6 ($p < 0.05$). Fewer B. vulgatus were cultured from these abscesses on days 6 and 21 compared with the numbers of viable B. fragilis MFN 1110 in abscesses induced by AIM ($p < 0.05$; Fig.3.3 cf. Fig.3.2). The ratio of viable B. vulgatus to viable E. coli six and 21 days post-inoculation was approximately 1:27. The numbers of viable E. coli in abscesses induced by B. vulgatus, E. coli and bran were not significantly different from those in abscesses induced by AIM on days 6 and 21 post-inoculation.

Abscesses induced by B. vulgatus, E. coli and bran were not significantly smaller than abscesses induced by B. fragilis ATCC 23745, E. coli and bran on days 6 and 21 (Table 3.2). However, on days 6 and 21 significantly fewer B. vulgatus than B. fragilis ATCC 23745 were isolated from the abscesses ($p < 0.05$; Fig.3.3).

Abscesses induced by the combinations of bacteria and bran in Table 3.2 were found in the peritoneal cavity in locations similar to abscesses induced by AIM.

3.2.3 Abscess Histology

The cellular organization of abscesses caused by B. fragilis MFN 1110, E. coli and bran was apparent by day 3. In Fig.3.4 the abscess is confined by a developing wall of granulation tissue containing some collagen, and beneath it is an area of mononuclear phagocytes. Neutrophils make up the rest of the abscess interior, and at the centre liquefactive necrosis is evident. As the abscesses aged, the main histological features were the further development and thickening of the wall of granulation tissue (Fig.3.5) and the enlargement of the necrotic centre. In 40 or 70 day old abscesses, the central necrotic area was a pink staining amorphous mass in haematoxylin-eosin stained sections (Fig.3.6). The region of mononuclear phagocytes did not appear to expand as the abscesses aged.

B. fragilis antigens were distributed throughout the central area of neutrophils and in the layer of mononuclear phagocytes immediately beneath the wall of the abscess (Fig.3.7). E. coli antigens were only found in the central area of neutrophils.

Some abscesses induced by a combination of either B. fragilis and E. coli or B. vulgatus, E. coli and bran were resolving six days post-inoculation. These abscesses did not have a defined wall and were composed of some neutrophils, mononuclear cells and fibroblasts.

Figure 3.4: Histology of a day 3 abscess induced by AIM. A section of an abscess stained by Gomori's one-step trichrome method to reveal collagen. The granulation tissue (gt) of the abscess wall contains some pale green collagen (arrowhead). Beneath the wall is an area of mononuclear phagocytes (mn). Neutrophils (pmn) comprise the rest of the abscess. A developing central necrotic zone (cnz) is evident.
x 78.75.

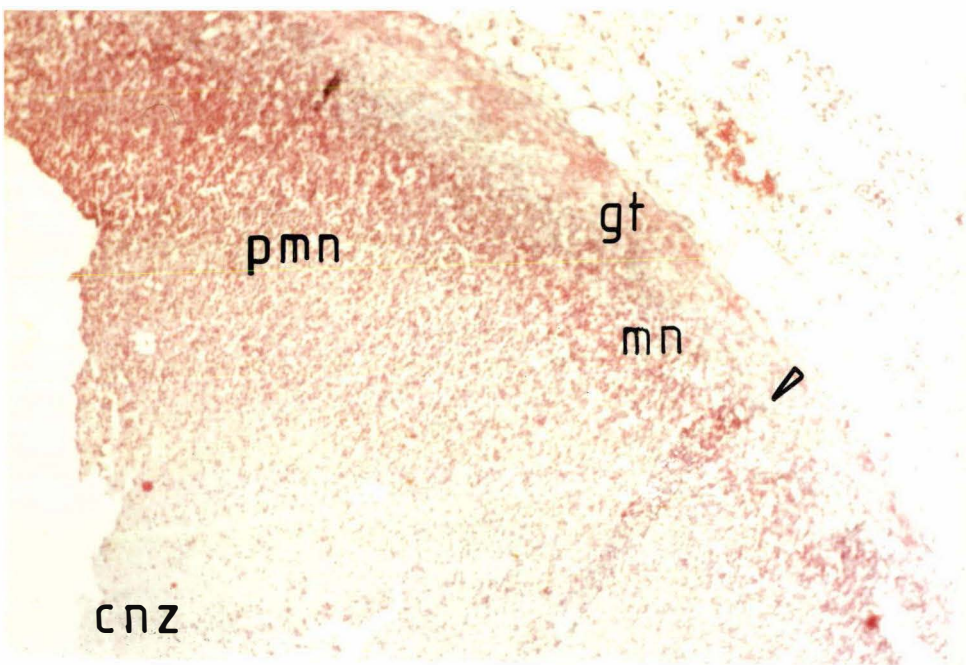
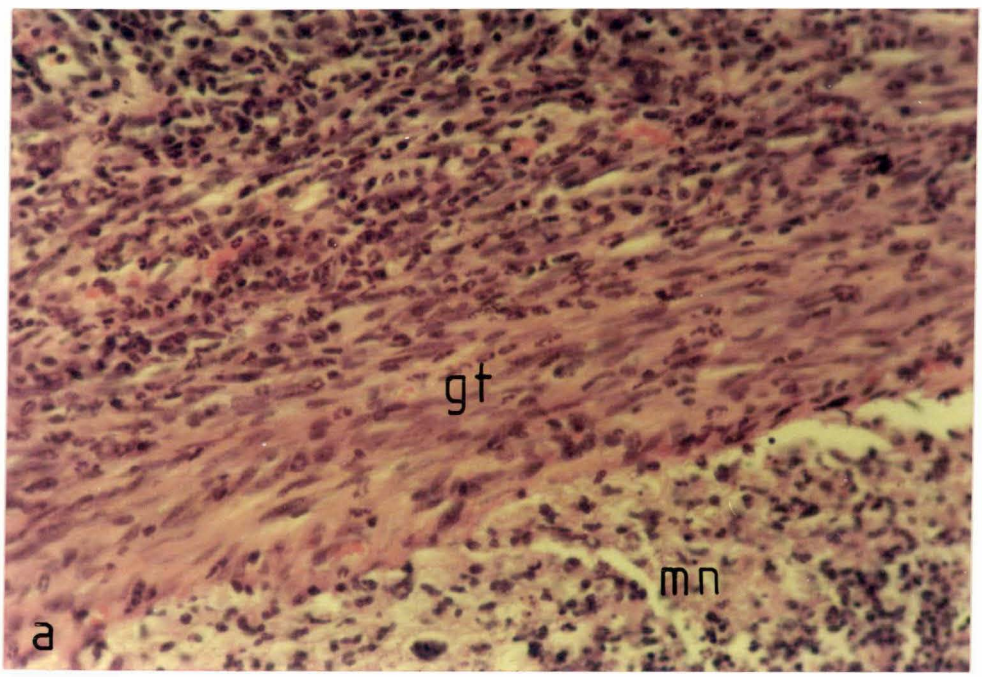

pmn
gt
mn
cnz

Figure 3.5: Histology of a day 13 abscess induced by AIM. A section of an abscess stained with haematoxylin-eosin (a) and Gomori's one-step trichrome method (b) showing the wall of granulation tissue (gt) and band of mononuclear phagocytes (mn). Green collagen fibres (arrowhead) can be seen in (b). (a) and (b) x 312.5.


gt
mn
a

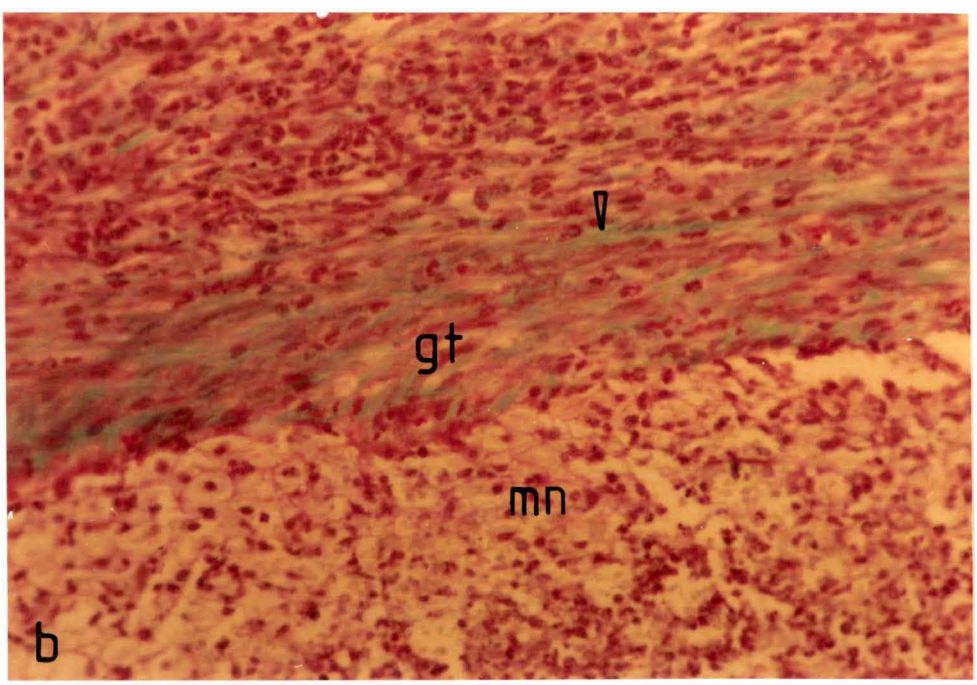
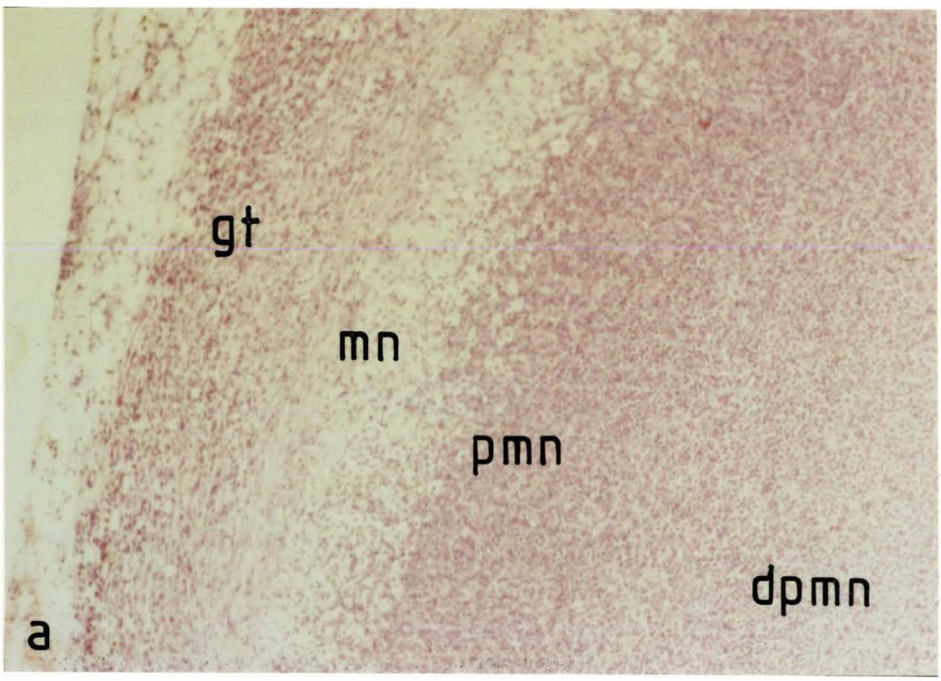

gt
mn
b

Figure 3.6: Histology of a day 40 abscess induced by AIM. A section of an abscess stained with haematoxylin-eosin. The wall of granulation tissue (gt), band of mononuclear phagocytes (mn) and area of neutrophils (pmn) are visible in (a). Further inside the abscess than (a) can be seen more degenerating neutrophils (dpmn) and the extensive central necrotic zone (cnz) as shown in (b).
(a) and (b) x 78.75.


gt
mn
pmn
dpmn
a

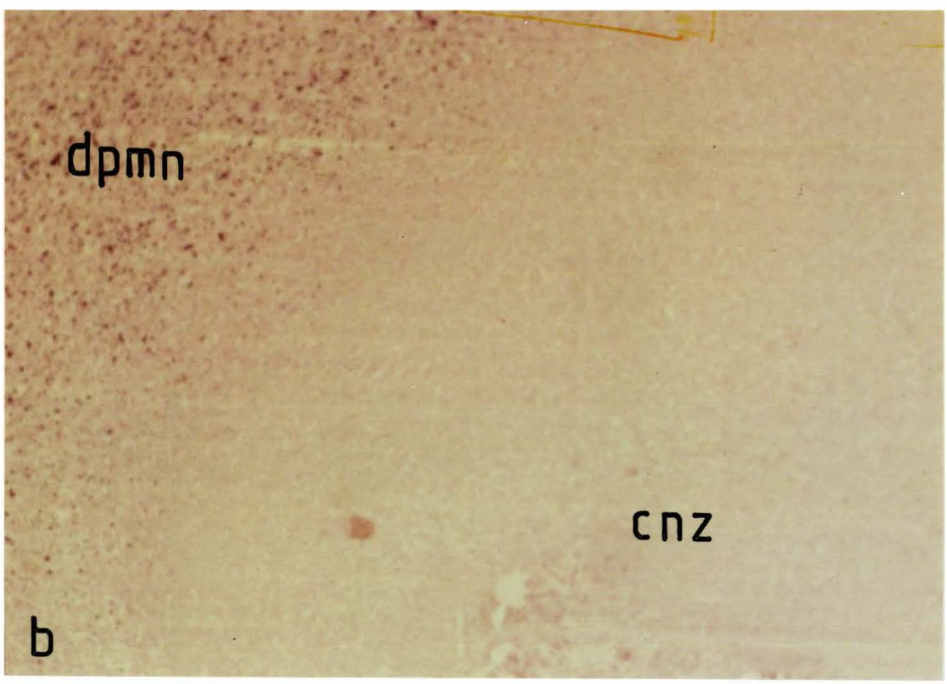
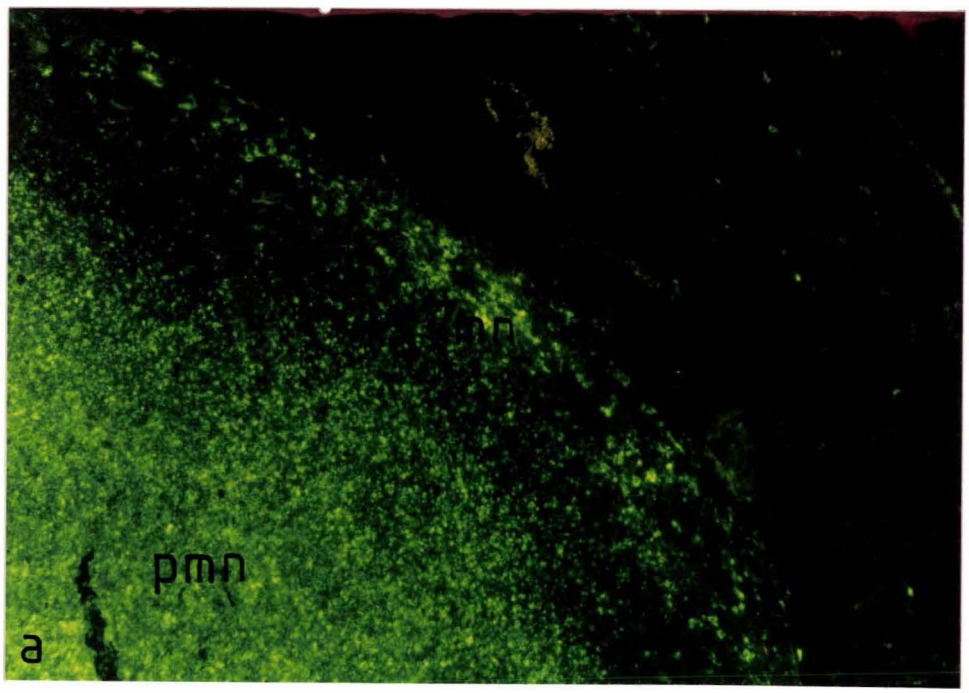
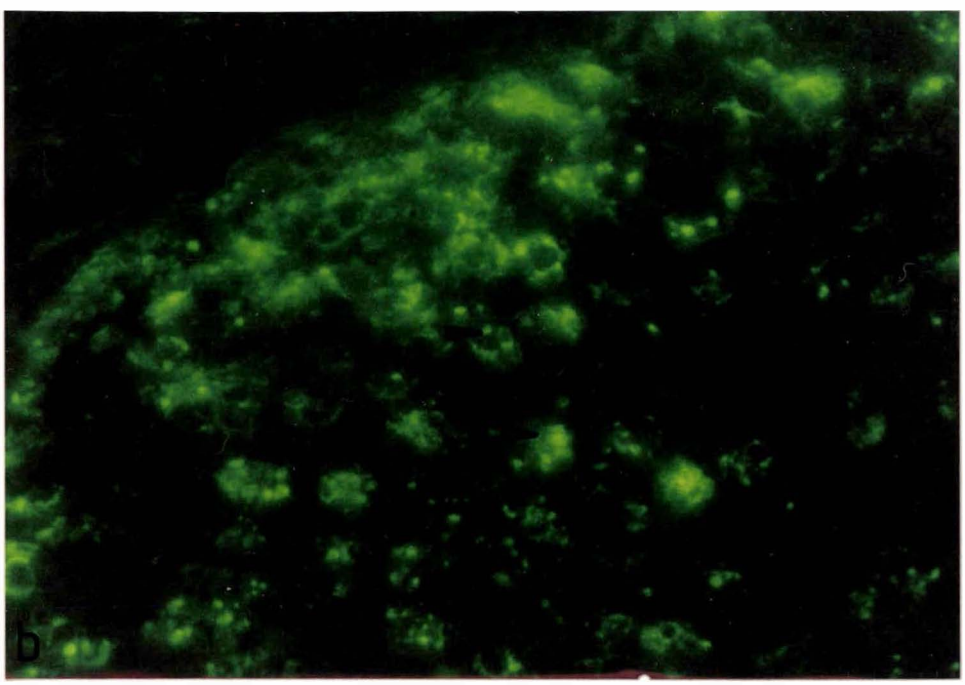

dpmn
cnz
b

Figure 3.7: Distribution of B.fragilis antigens within abscesses induced by AIM. Three (a) or 13 (b) days after inoculation of mice with AIM, abscesses were removed from mice, fixed, sectioned and stained with anti-B. fragilis serum by an indirect fluorescent antibody method.

(a) B. fragilis antigens are visible in the layer of mononuclear phagocytes (mn) immediately beneath the abscess wall and in the central area of neutrophils (pmn).
x 39.69.

(b) B. fragilis antigens can be seen within individual phagocytes (arrowheads) in the area beneath the abscess wall of granulation tissue (gt).
x 252.


pmn
a


b

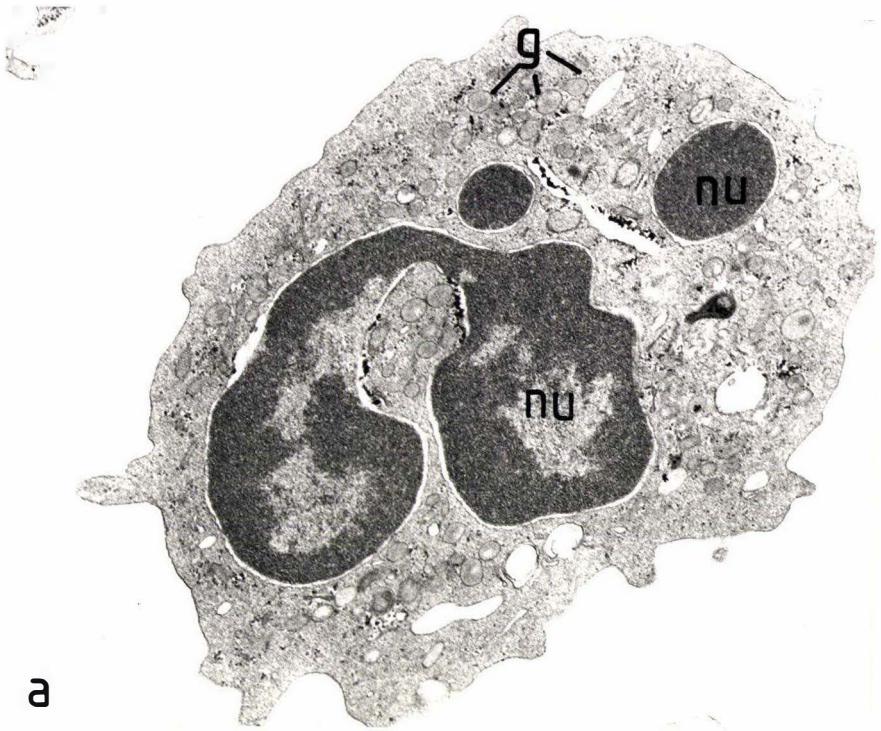
3.2.4 Ultrastructure of Murine Peritoneal Neutrophils

Murine peritoneal neutrophils have a multi-lobed nucleus and a dense cytoplasm (Fig.3.8a). Smooth or rough endoplasmic reticulum, mitochondria and Golgi bodies were rarely seen in the cytoplasm of mature neutrophils. Glycogen granules were seen in the cytoplasm of some cells. The most prominent structures within a neutrophil's cytoplasm were the primary (azurophilic) and secondary (specific) granules (Fig.3.8b). The granules showed heterogeneity in their size and shape. The primary granules were identified by labelling the neutrophils for peroxidase activity, since the primary granules contain myeloperoxidase. Peroxidase-positive primary granules and peroxidase-negative secondary granules can be seen in Figs.3.9 and 3.10. The peroxidase-positive granules were as varied in size and shape as the peroxidase-negative granules. There were more unlabelled than labelled granules in the cytoplasm of neutrophils. Control neutrophils which had been incubated in Graham's and Karnovsky's medium from which 3-3' diaminobenzidine tetrahydrochloride and hydrogen peroxide had been omitted had unlabelled granules (Fig.3.11).

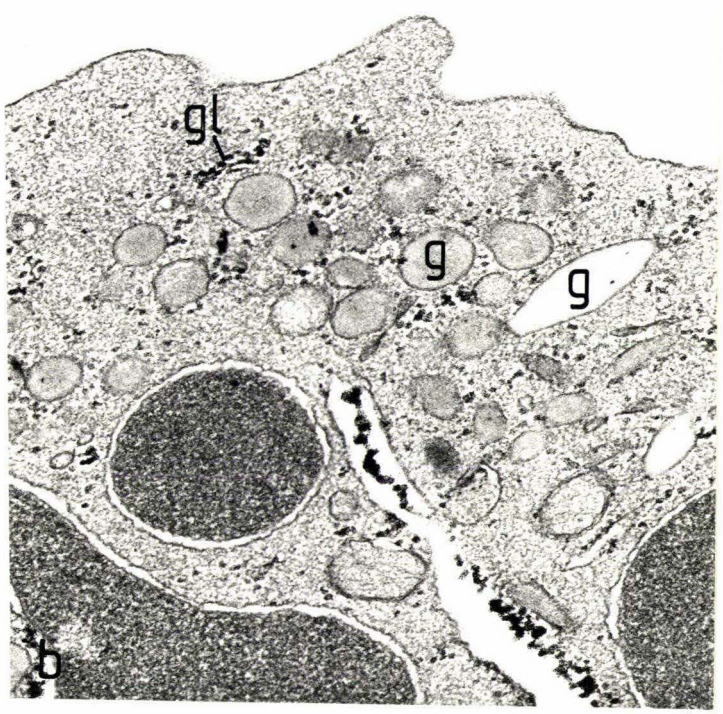
Periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) staining of murine peritoneal neutrophils was evaluated as a method of identifying the secondary granules. In Fig.3.12 glycogen within the neutrophil's cytoplasm has been stained by the PA-TCH-SP reaction which obscures the granules. However, PA-TCH-SP staining of neutrophils after α-amylase digestion of the glycogen revealed the stained granules (Fig.3.13). PA-TCH-SP positive granules generally had a stained interior and surrounding membrane. Fig.3.14 shows no staining of cytoplasmic granules was observed in control

Figure 3.8: (a) A mature neutrophil from the murine peritoneal cavity. The cell has the characteristic multi-lobed nucleus (nu) and a dense cytoplasm containing many granules (g). Other cytoplasmic organelles are not visible. No peroxidase label.
x 18579.

(b) Some cytoplasmic granules (g) of (a) enlarged. The granules exist in a range of sizes and shapes. Some glycogen particles (gl) are visible.
x 49989.



a

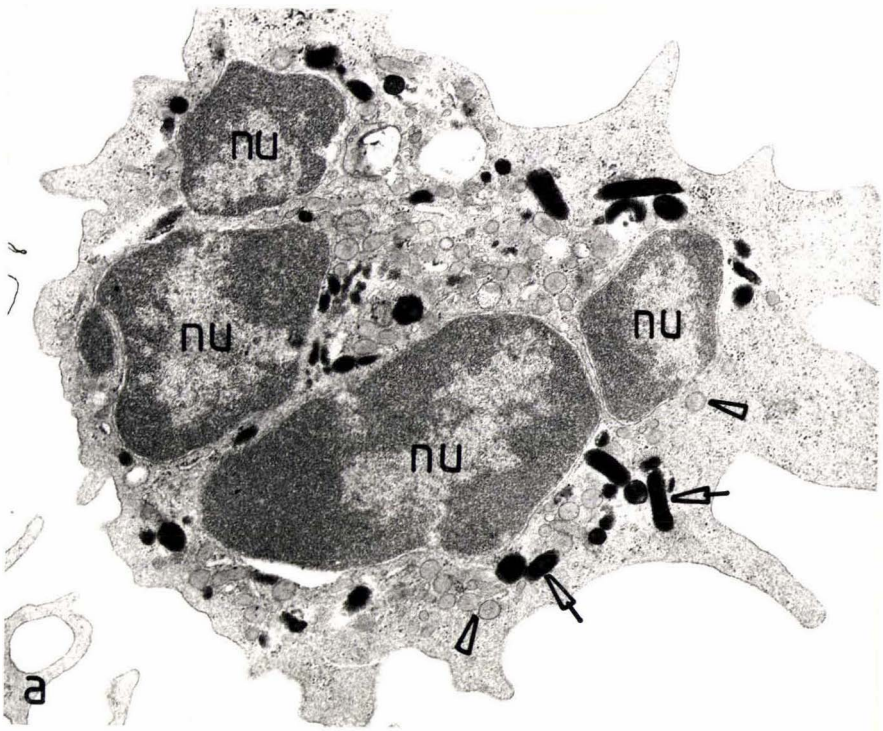


b

Figure 3.9: (a) A peroxidase-labelled mature neutrophil from the murine peritoneal cavity. The peroxidase-positive primary granules (arrows) are outnumbered by the peroxidase-negative secondary granules (arrowheads). All granules are varied in size and shape. nu = neutrophil nucleus. x 18579.

(b) Enlargement of some cytoplasmic granules of (a). x 49989.

Control neutrophils are shown in Fig.3.11.


nu
nu
nu
nu
a

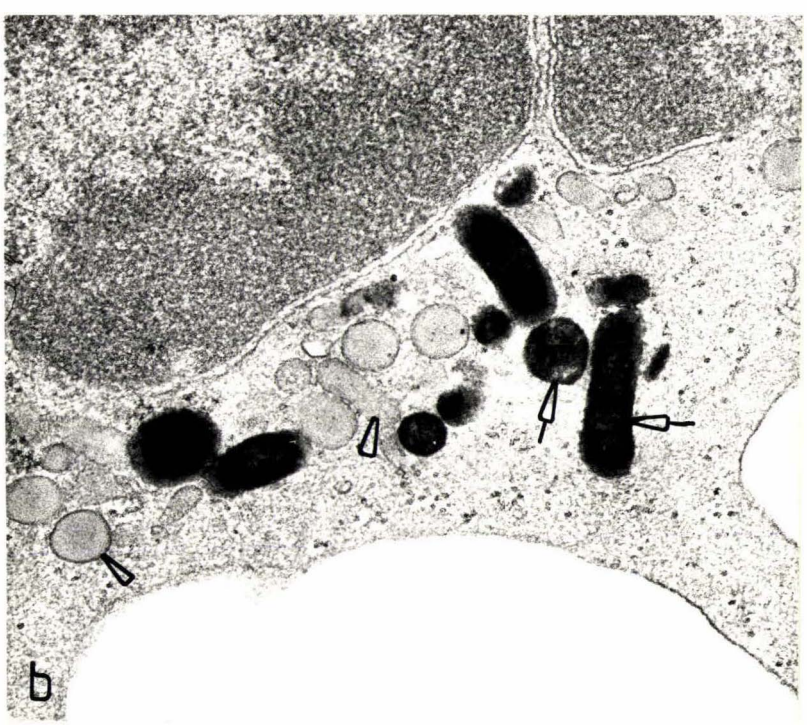
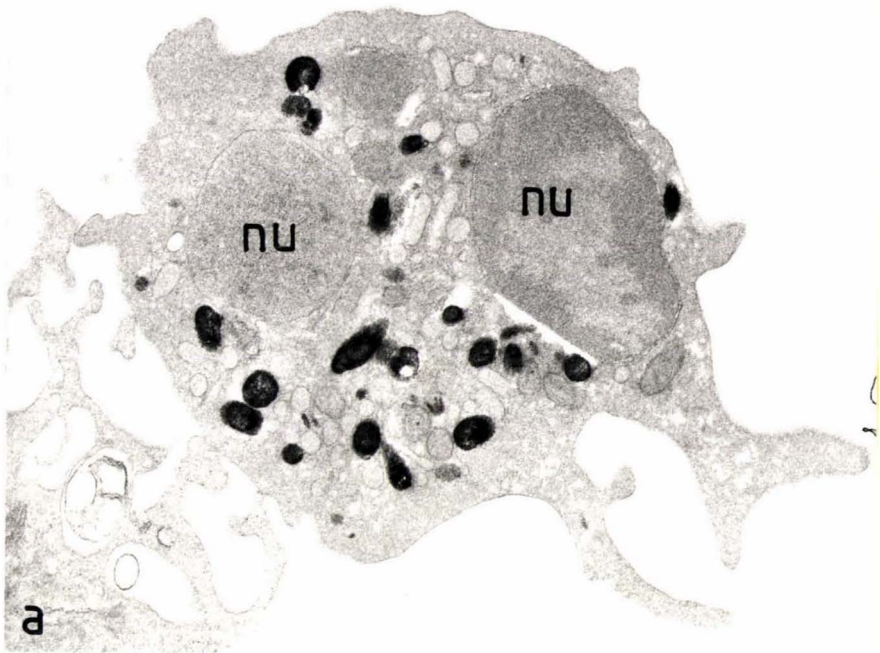

b

Figure 3.10: (a) A peroxidase-labelled mature neutrophil from the murine peritoneal cavity. The section was not stained with lead citrate to illustrate the specificity of the peroxidase labelling for the primary granules.
nu = neutrophil nucleus.
x 18579.

(b) Enlargement of some cytoplasmic granules of (a). Peroxidase-positive (arrow) and peroxidase-negative (arrowhead) granules are visible.
x 49989.


nu
nu
a

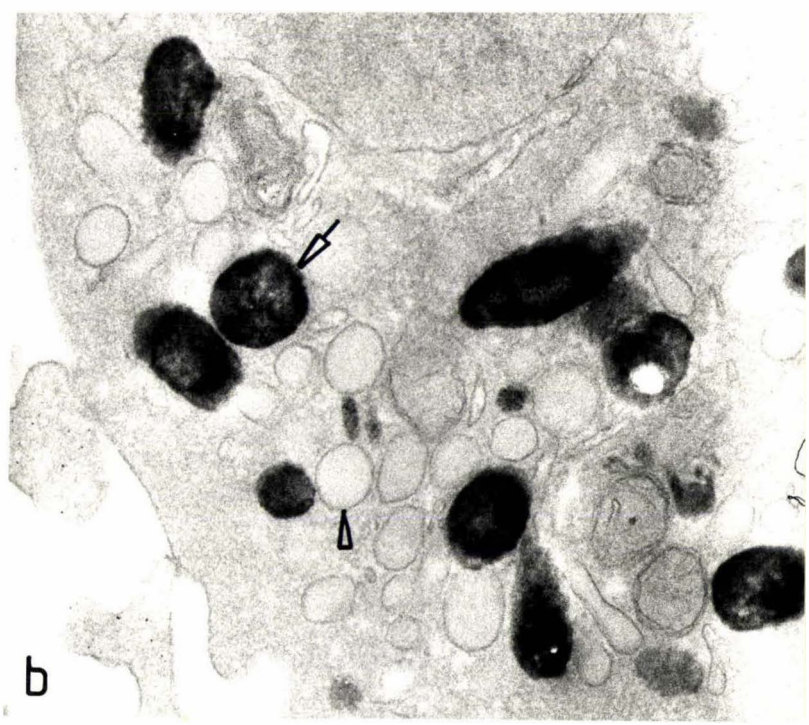
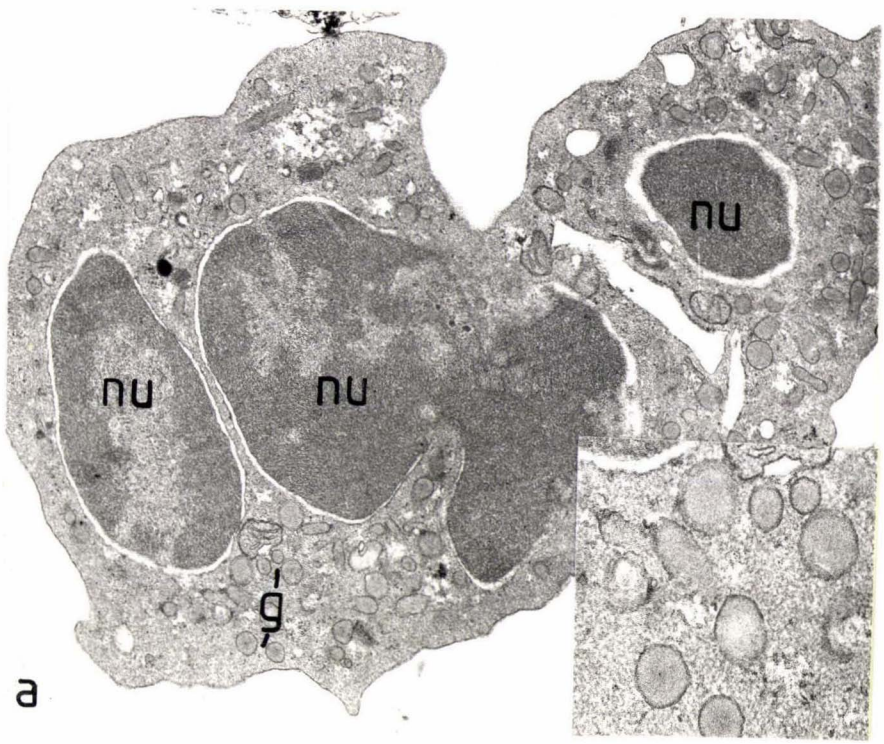
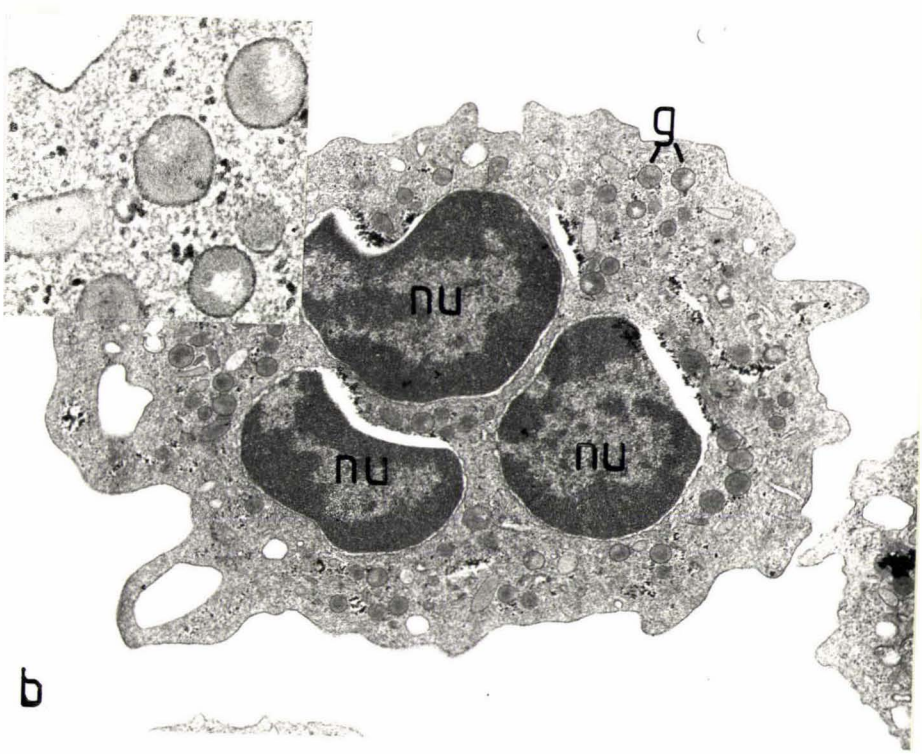

b

Figure 3.11: (a) Mature peritoneal neutrophil which was incubated in Graham's and Karnovsky's medium for peroxidase from which H_2O_2 was omitted. The granules (see inset) are not labelled for peroxidase activity.
x 18579; inset x 48600.

(b) Mature peritoneal neutrophil which was incubated in Graham's and Karnovsky's medium for peroxidase from which 3-3' diaminobenzidine tetrahydrochloride was omitted. The granules (see inset) are not labelled for peroxidase activity.
x 14400; inset x 48600.

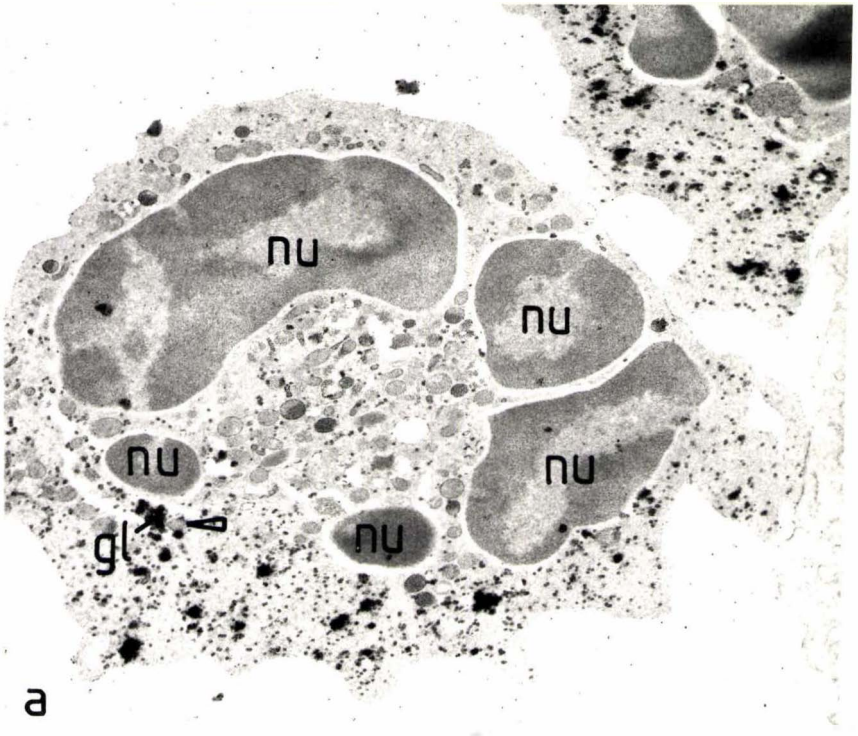
nu = neutrophil nucleus
g = granules


nu
nu
nu
g
a


g
nu
nu
nu
b

Facing Page 77

Figure 3.12: (a) A PA-TCH-SP stained mature peritoneal neutrophil. Stained glycogen granules (gl) mask the stained granules (arrowhead) which are shown enlarged in (b). nu = neutrophil nucleus. x 18579; (b) x 49989.



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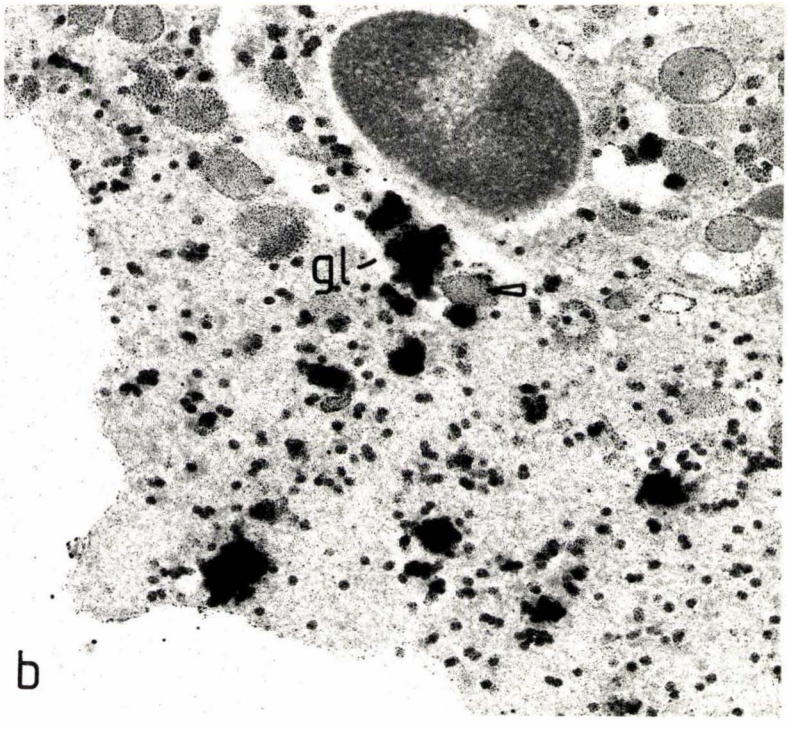
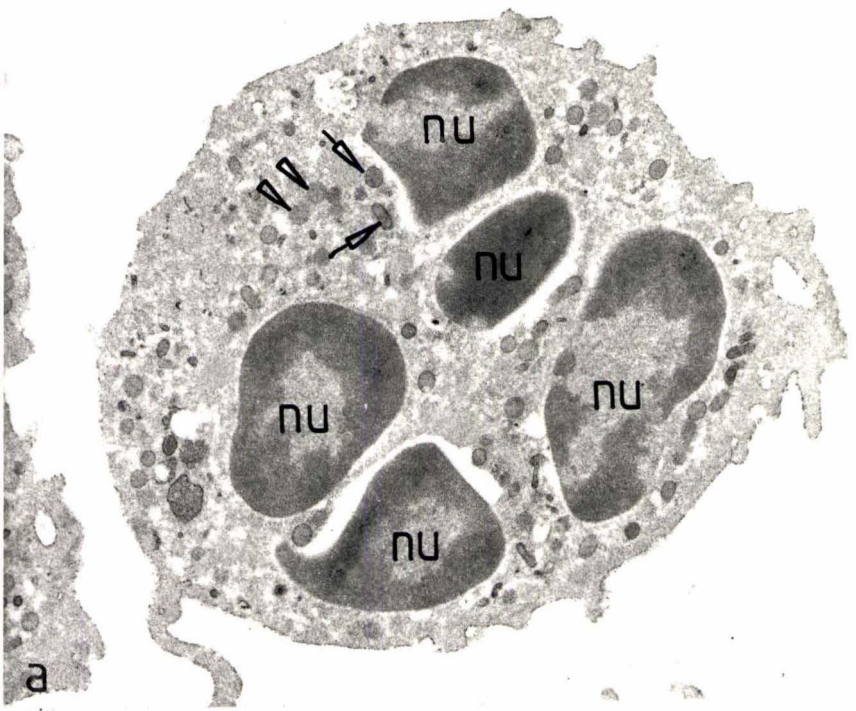


b

Figure 3.13: (a) A PA-TCH-SP stained mature peritoneal neutrophil which has been digested with α-amylase to remove the glycogen. Intensely stained granules (arrows) and weakly stained granules (arrowheads) are shown enlarged in (b).
nu = neutrophil nucleus.
x 18579; (b) x 49989.

Control neutrophils are shown in Fig.3.14.


nu
nu
nu
nu
nu
a

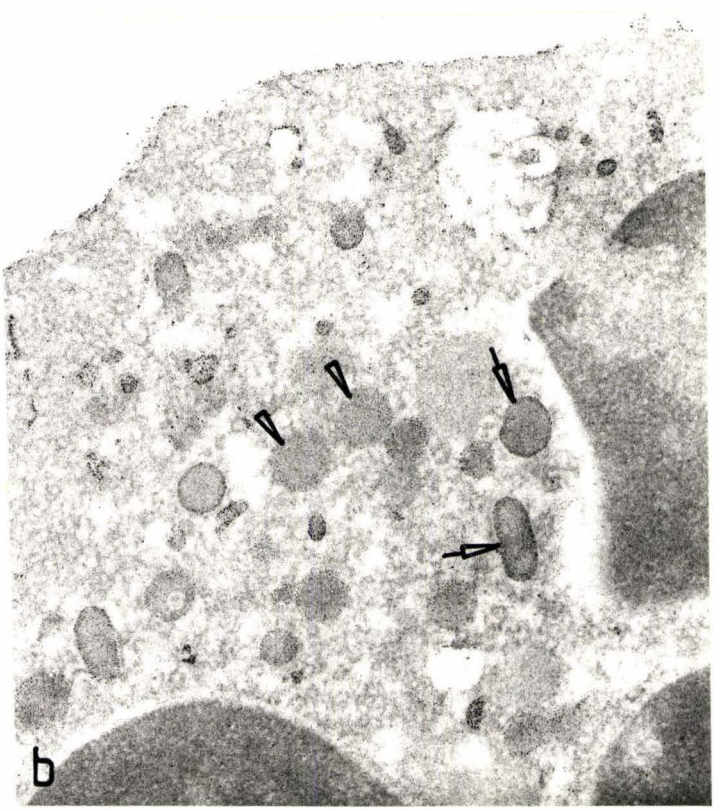

b

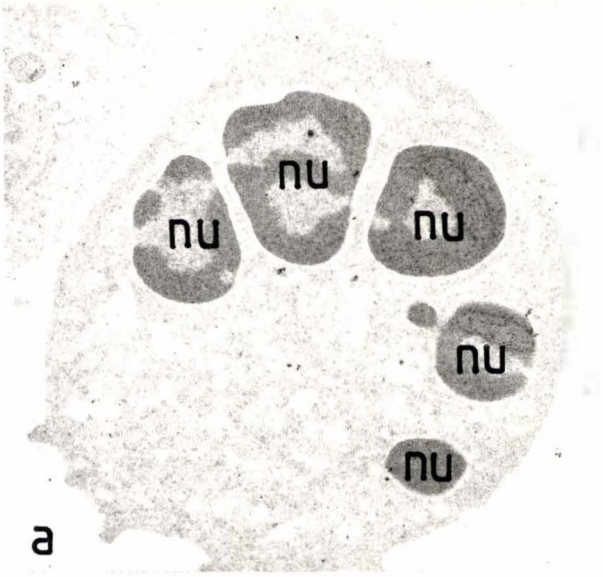
Figure 3.14: Control mature peritoneal neutrophils which illustrate that PA-TCH-SP positive granules contain vicinal glycol-containing glycoconjugates. No reactive granules are visible in the control neutrophils which were stained as follows:

(a) Thiocarbohydrazide - silver proteinate stained, x 14207,

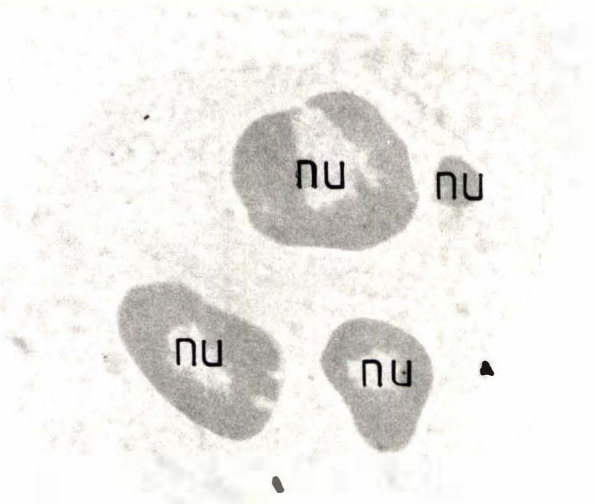
(b) Periodate - silver proteinate stained, x 14207,

(c) Silver proteinate stained, x 14207.

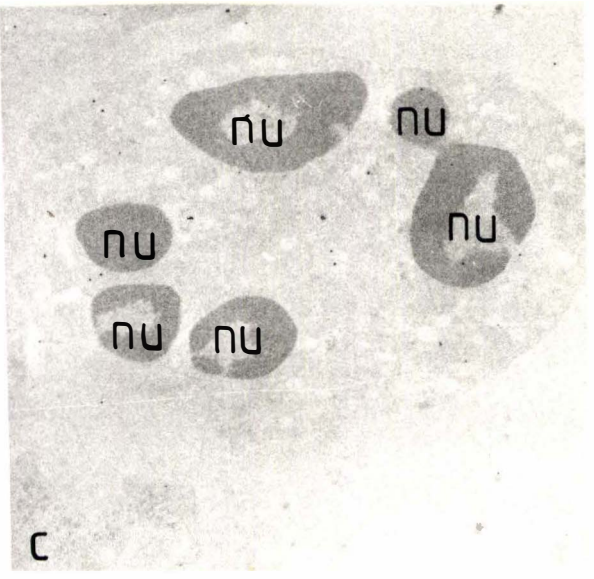
nu = neutrophil nucleus



a



b



c

sections (thiocarbohydrazide-silver proteinate, periodate-silver proteinate and silver proteinate stained) indicating the PA-TCH-SP positive granules contained vicinal glycol-containing glycoconjugates. Thus a population of PA-TCH-SP positive granules existed within neutrophils, but the variety in the intensity of staining observed did not allow accurate discrimination between primary and secondary granules.

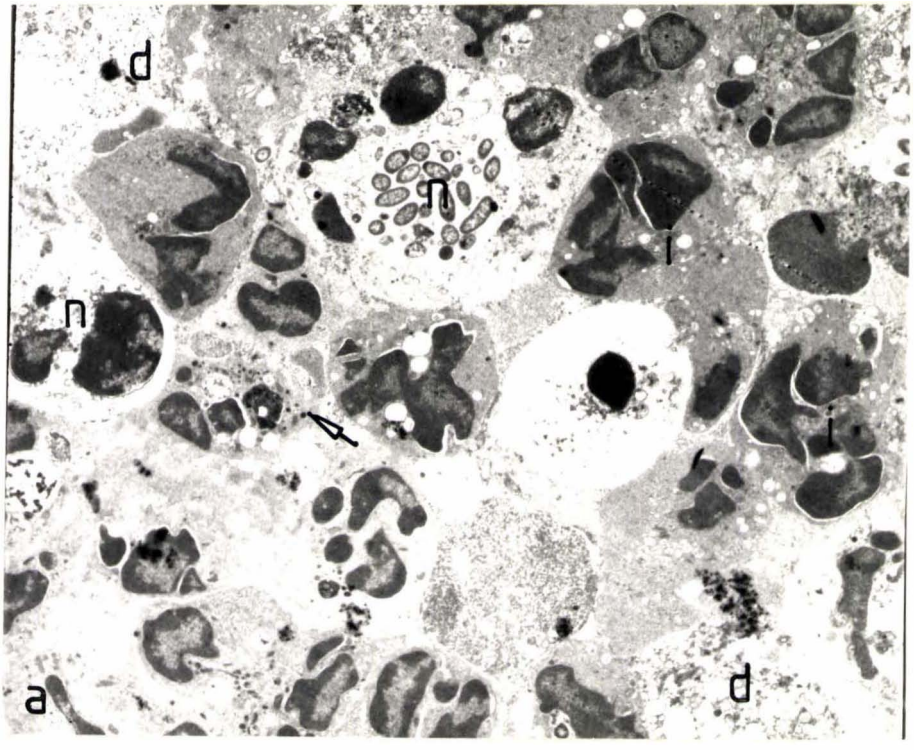
3.2.5 Abscess Ultrastructure

The ultrastructure of abscesses induced by AIM was studied to observe the neutrophils and other abscess components and the location of the bacteria as the abscesses aged. Three days post-inoculation with AIM, the interior of the abscesses was composed of a mixture of intact and necrotic neutrophils, plus necrotic cellular debris (Fig.3.15a). Cytoplasmic granules were seen in some intact neutrophils. Some necrotic neutrophils had large phagosomes containing debris, and in some instances many bacteria (Fig.3.15b). By day 6 the abscess interior contained more necrotic neutrophils and debris, although intact neutrophils with cytoplasmic granules were seen (Fig.3.16a). Some of these neutrophils have pseudopodia extended, which suggests cell movement. The neutrophil in Fig.3.16b has phagosomes which contain debris and a bacterium. Granules are seen in this neutrophil's cytoplasm. Fig.3.16c shows an example of the blood vessels and collagen which form part of the granulation tissue of the abscess wall by day 6. Mononuclear cells are visible in the lumena of the blood vessels. Fig.3.17a shows the interior of a 13 day old abscess between the abscess wall and the abscess centre where neutrophils are surrounded by cellular debris. Some bacteria can be seen amongst the debris. The neutrophils are degenerating, as indicated by the decreased electron density of their cytoplasm. The neutrophils'

Figure 3.15: Ultrastructure of a day 3 IA abscess induced by AIM

(a) General view of the abscess interior which consists of a mixture of intact (i) and necrotic (n) neutrophils, plus necrotic cellular debris (d). Cytoplasmic granules (arrow) are visible in some neutrophils. x 4129.

(b) Enlargement of a necrotic neutrophil labelled in (a). The multi-lobed nucleus (nu) is visible, plus the neutrophil's cell membrane (arrowheads). The cytoplasm (c) has lost some electron density. A large phagosome (p) containing bacteria (b), most of which appear intact, is visible. x 9806.


d
n
n
i
i
d
a

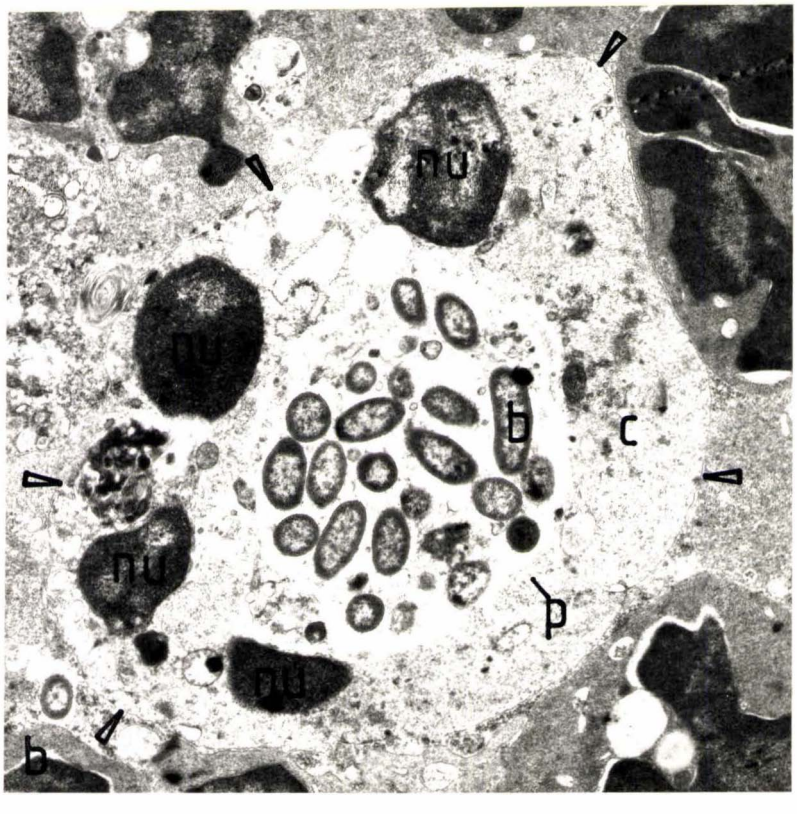
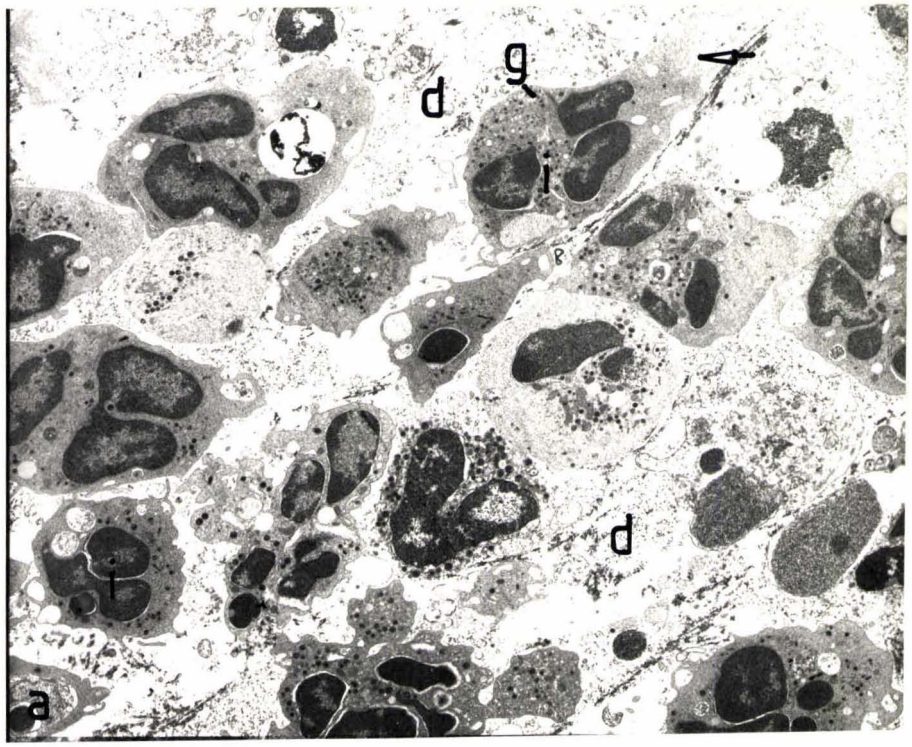

nu
nu
b
c
nu
p
nu
b

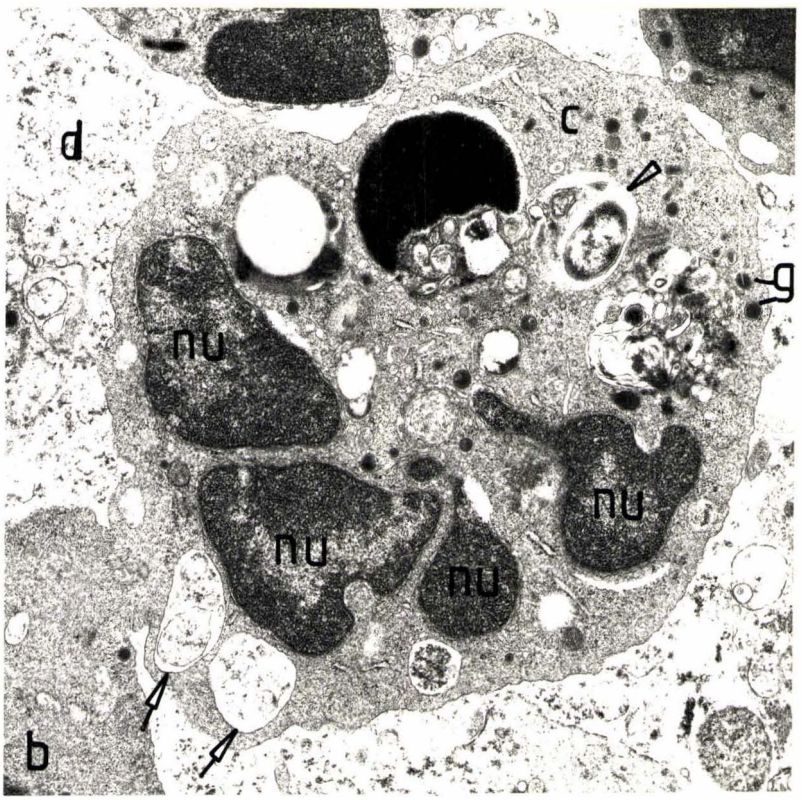
Figure 3.16: Ultrastructure of a day 6 IA abscess induced by AIM

(a) General view of the abscess interior which consists of more necrotic debris (d), although intact neutrophils (i) are visible. The intact neutrophils have many granules (g) in their cytoplasm. Some neutrophils have pseudopodia extended (arrow), which suggests directed movement.
x 4129.

(b) A neutrophil amongst necrotic debris (d) in the abscess interior. The cytoplasm (c) is electron dense indicating the cell is probably not degenerate. Some cytoplasmic granules (g) are visible. The neutrophil contains several phagosomes, of which some contain debris (arrows) and one contains a bacterium (arrowhead).
nu = neutrophil nucleus.
x 12800.

(c) Blood vessels (arrows) in the granulation tissue (gt) of the abscess wall. The blood vessels contain red blood cells (rbc) and mononuclear cells (mn). Collagen (arrowhead) is visible in the granulation tissue. x 3497.


g
d
i
d
i
a


d
c
g
nu
nu
nu
nu
b

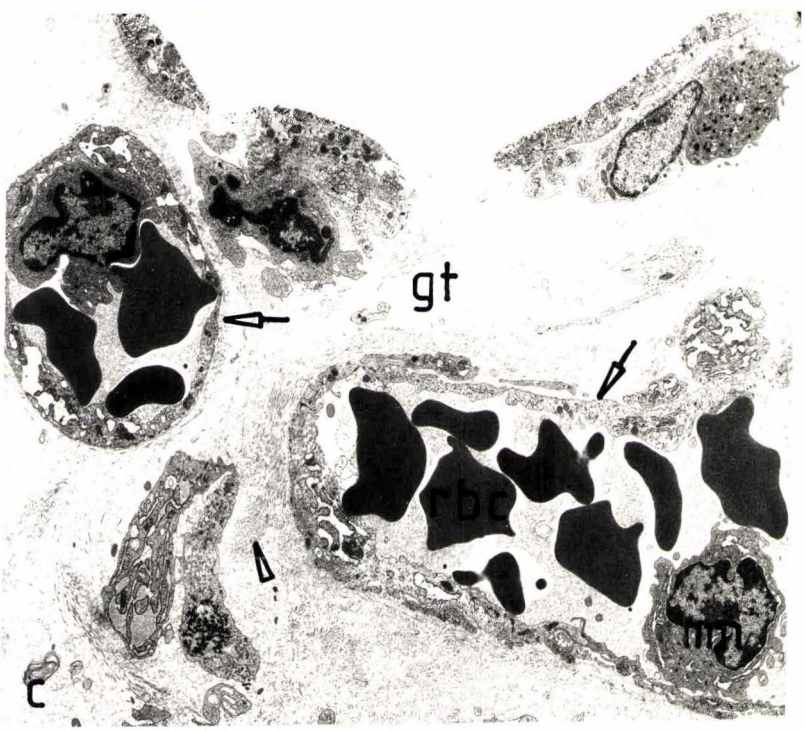

gt
rbc
mn
c

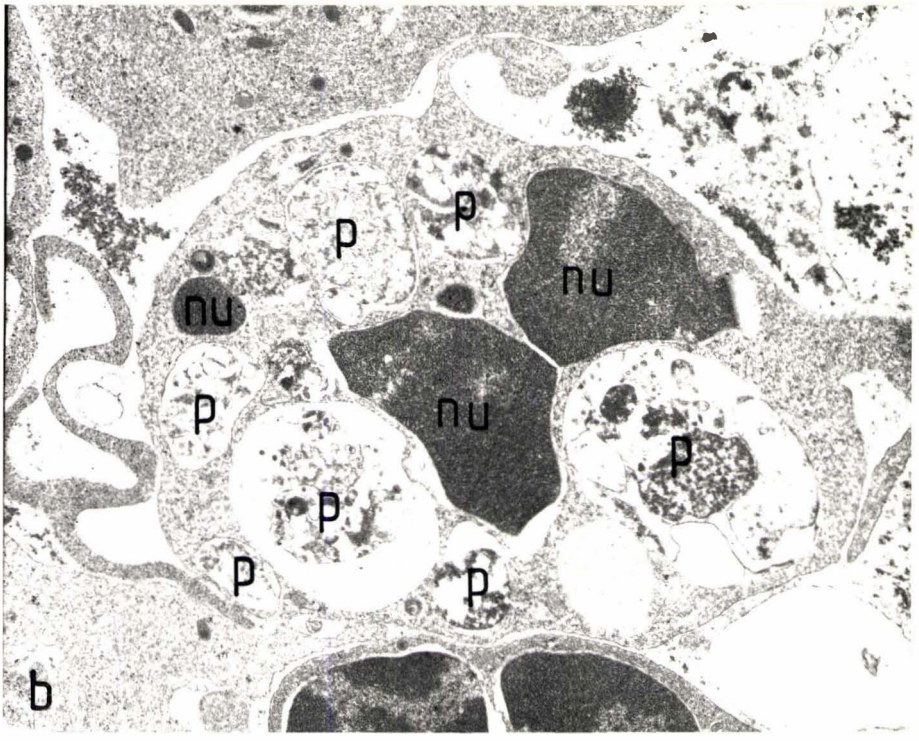
Figure 3.17: Ultrastructure of a day 13 abscess induced by AIM

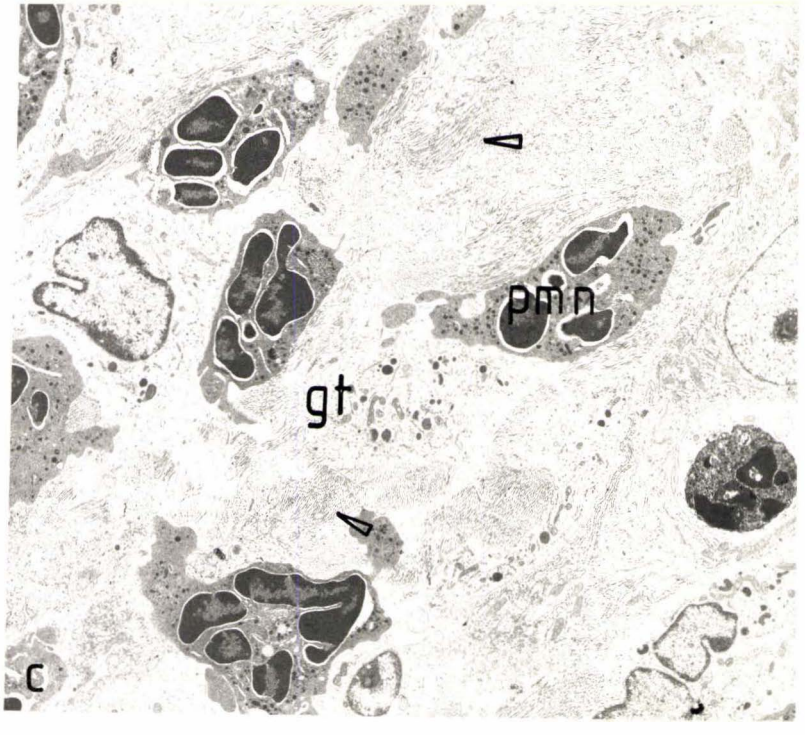
(a) General view of the abscess interior which shows extensive necrotic debris (d) and neutrophils (arrows). Some extracellular bacteria (arrowheads) are visible.
x 4129.

(b) An example of a neutrophil within the abscess interior. The cytoplasm contains phagosomes (p) filled with debris. No cytoplasmic granules are visible.
nu = neutrophil nucleus.
x 14400.

(c) The well developed granulation tissue (gt) contains much collagen (arrowheads). Migrating intact neutrophils (pmn) are visible.
x 3497.

a
d


p
p
nu
nu
p
nu
p
p
p
p
p
b


pmn
gt
c

phagosomes contain material of bacterial and possibly cellular origin (Fig.3.17b). The walls of 13 day old abscesses had well developed granulation tissue, as indicated by the presence of collagen. Incoming intact neutrophils were seen in the granulation tissue (Fig.3.17c). By day 21 many abscess neutrophils had phagocytosed a lot of necrotic debris (Fig.3.18a) and in some instances the purulent exudate was composed of bacteria amongst debris and fragments of pyknotic neutrophil nuclei (Fig.3.18b). This outcome of liquefactive necrosis was also apparent in abscesses at days 40 (Fig.3.19a) and 70 (Fig.3.20). By day 40 the granulation tissue enclosing the purulent exudate was very dense, with much collagen in evidence (Fig.3.19b). Mononuclear phagocytes were not observed in the interior of the abscesses examined at various intervals post-inoculation with AIM, but were occasionally seen in the wall area of abscesses (Fig.3.16c).

In one experiment, abscesses which had been fixed and embedded in paraffin wax for histological purposes were dewaxed and subjected to further processing for electron microscopy studies. This enabled the examination of the ultrastructure of histological features observed in haematoxylin-eosin stained sections. The technique enabled the abscess wall and the area beneath it to be identified (Fig.3.21). In some electron micrographs, bacteria were evident in this area but the location of the bacteria intra- or extra-cellularly could not be determined due to the poor preservation of ultrastructure obtained with this technique.

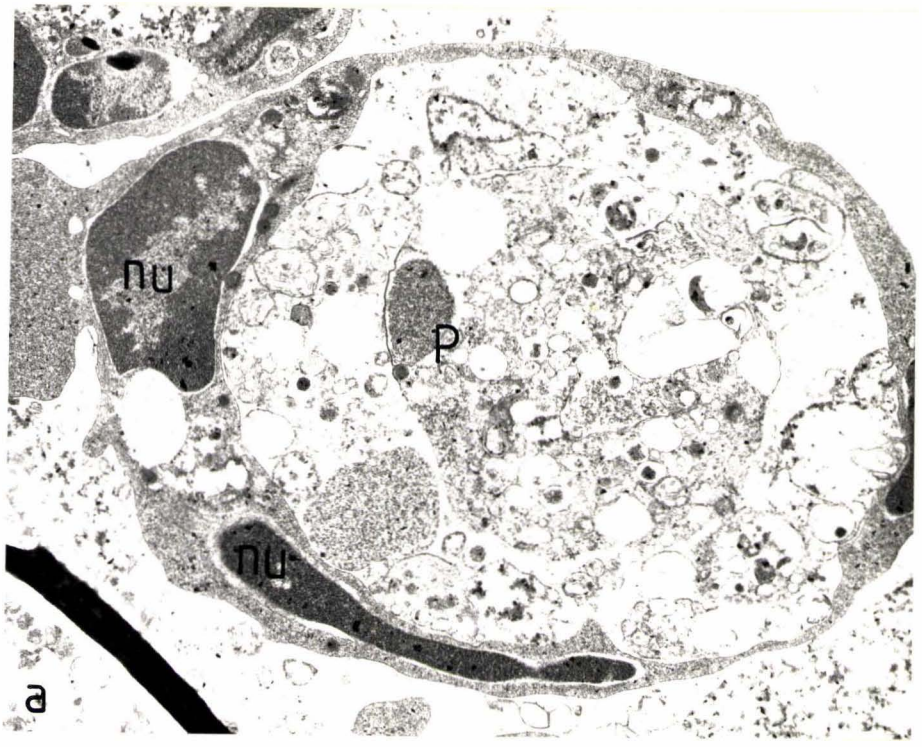
3.2.6 Initiation of Abscess Development

1 hr post-inoculation IP with *B. fragilis* MFN 1110 or *B. vulgatus* combined with *E. coli* or *E. coli* and bran, each group of mice had similar numbers of peritoneal leukocytes per mouse

Figure 3.18: Ultrastructure of a day 21 abscess induced by AIM

(a) A degenerating neutrophil in the abscess interior containing one large phagosome (P) filled with necrotic debris.
nu = neutrophil nucleus.
x 14400.

(b) General view of the abscess interior. Extracellular bacteria (B) can be seen amongst the necrotic debris (d) and pyknotic neutrophil nuclei (nu). x 9471.


nu
P
nu
a

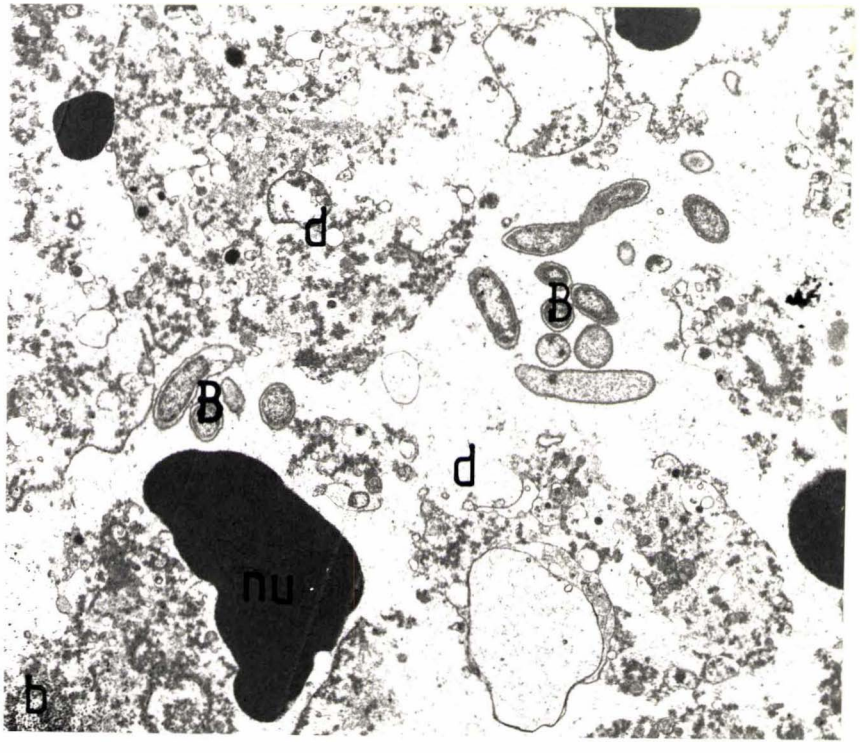
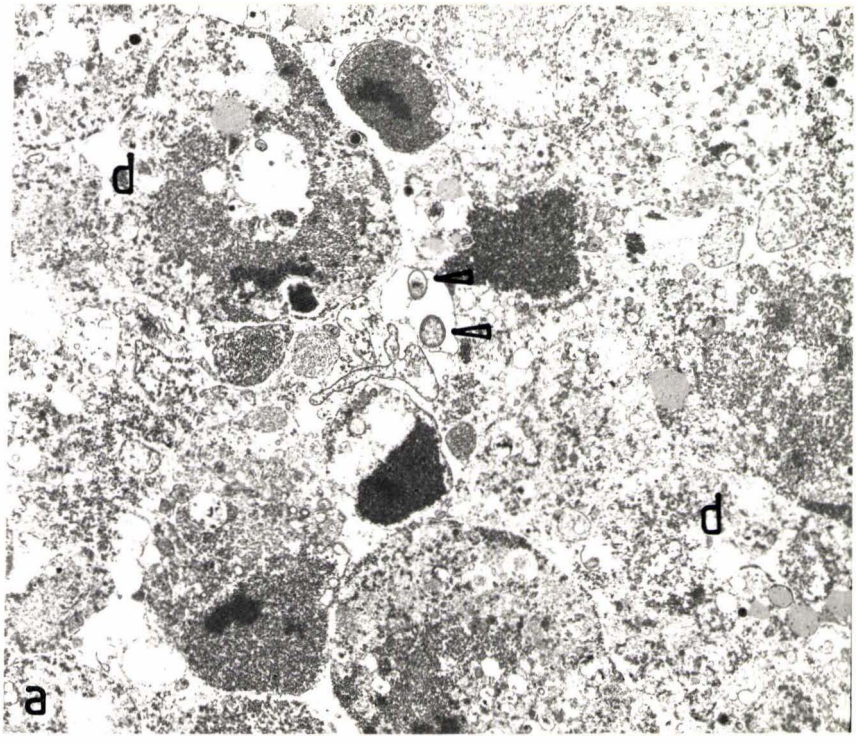

d
B
B
d
nu
b

Figure 3.19: Ultrastructure of a day 40 abscess induced by AIM

(a) General view of the necrotic debris (d) which forms the abscess interior. Extracellular bacteria (arrowheads) are visible. x 6314.

(b) Dense granulation tissue of the abscess wall which contains much collagen (co). x 6314.


d
d
a

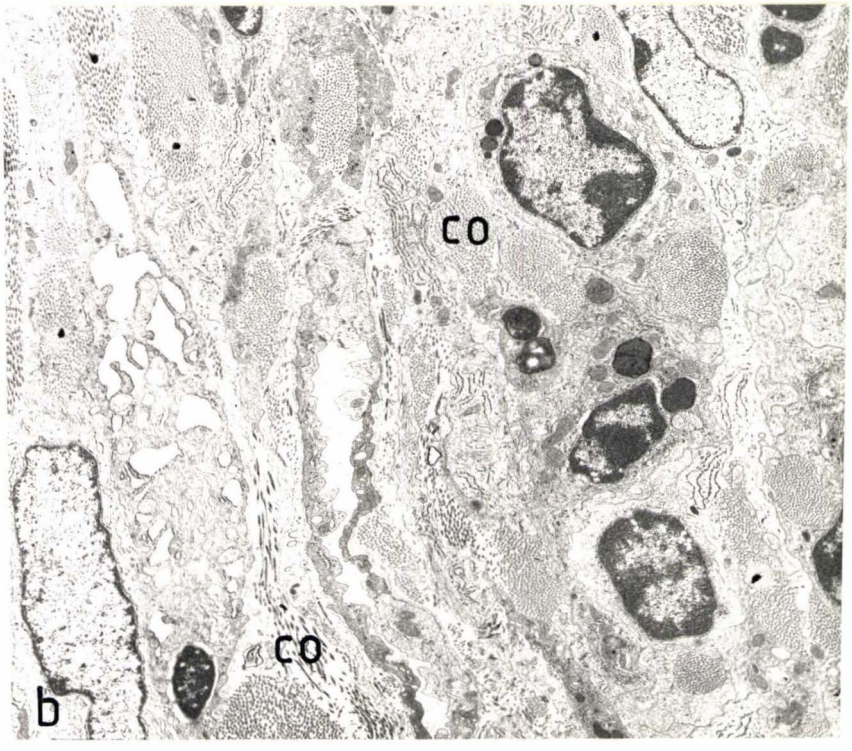

co
co
b

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Figure 3.20: Ultrastructure of a day 70 abscess induced by AIM

General view of extracellular bacteria (B) amongst the necrotic debris of the abscess interior. The necrotic remains of the neutrophils (pmn) can be discerned. x 9471.

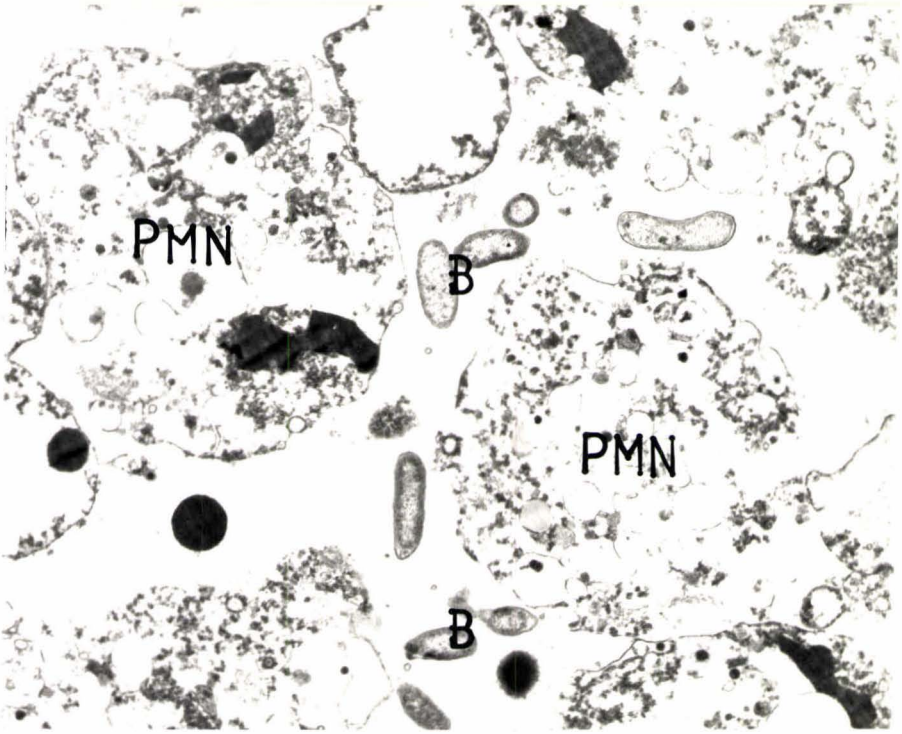
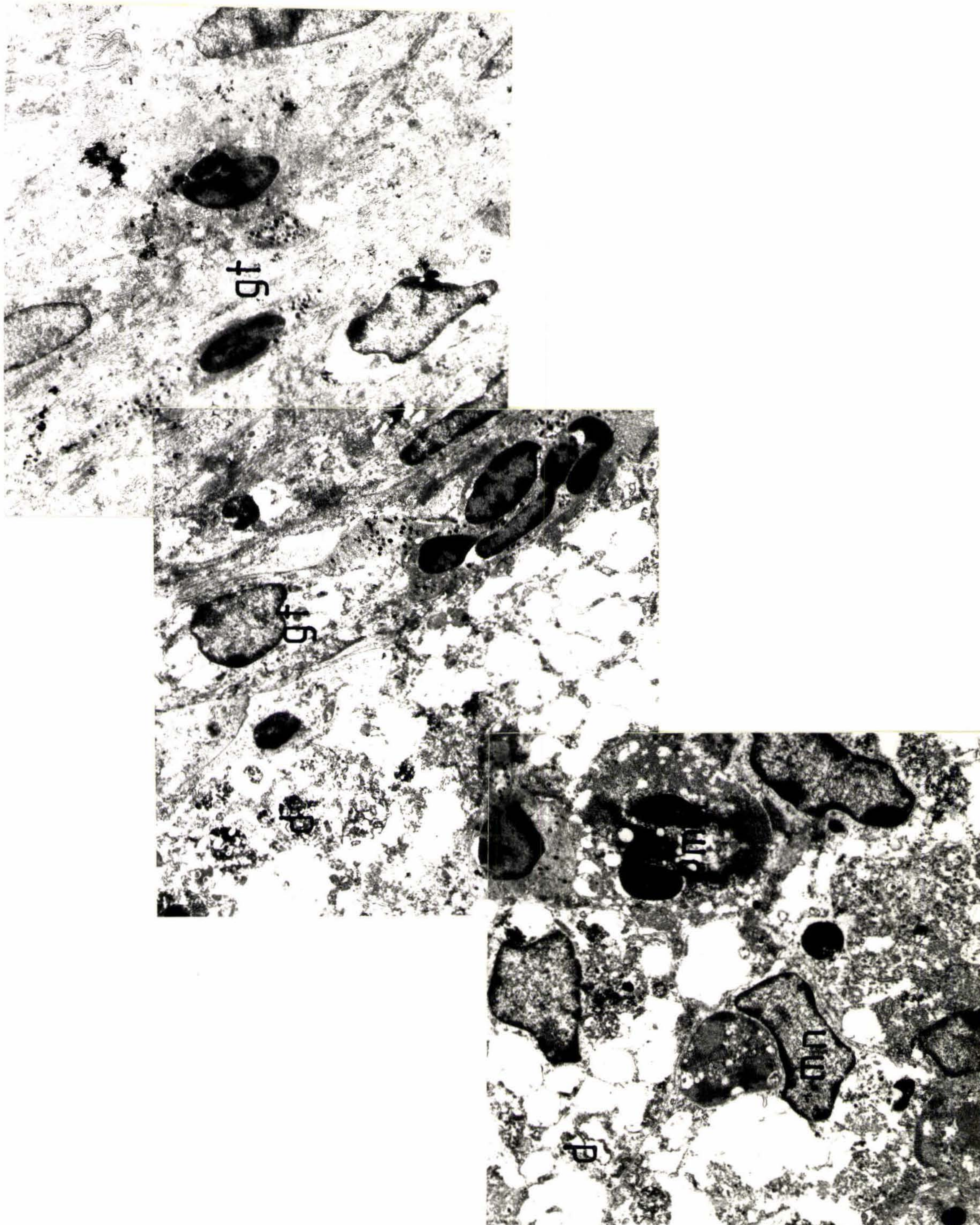

PMN
B
PMN
B

Figure 3.21: Reprocessing of histologically-fixed day 13 IA abscess induced by AIM for electron microscopy. The collage shows the granulation tissue (gt) of the abscess wall. A damaged neutrophil (pmn) and remnants of mononuclear cells identified by their nuclei (mn) are seen amongst the debris (d) immediately beneath the abscess wall. x 4829.



(Table 3.3), of which approximately 29% were neutrophils and 47% were mononuclear phagocytes (Table 3.4). There was a 4-6 fold increase in the number of peritoneal leukocytes per mouse 3.5 hrs later (Table 3.3) and many were neutrophils (Fig.3.23). Although mice given inocula containing bran had higher numbers of peritoneal leukocytes, the differences between these animals and those inoculated with bacteria alone were not significant 4.5 hrs after inoculation. After 24 hrs, all groups of mice had similar, slightly elevated, peritoneal leukocyte numbers.

Table 3.4 shows that 1 hr post-inoculation with the abscess-inducing mixtures, a greater percentage of neutrophils had phagocytosed bacteria than had mononuclear phagocytes. In the four groups of mice, the phagocytosis of the bacteria by neutrophils was similar in terms of the percentage of neutrophils with bacteria, and the ratio of bacteria to neutrophils. Although a smaller percentage of mononuclear phagocytes had phagocytosed bacteria, some cells had ingested many bacteria. Neutrophils and macrophages with leukocyte-associated bacteria 1 hr post-inoculation are shown in Fig.3.22. After 4.5 hrs had elapsed, less intracellular bacteria were seen in neutrophils and macrophages (Fig.3.23). 24 hrs post-inoculation intact neutrophils were seen, but many macrophages were vacuolated (Fig.3.24).

B. fragilis, *B. vulgatus* and *E. coli* were cultured from the washed peritoneal leukocytes during the 24 hrs following the IP inoculation with the abscess-inducing mixtures (Fig.3.25). The inoculation of bran with the bacteria resulted in significantly more intracellular *B. fragilis* and *E. coli*, but not *B. vulgatus*, after 4.5 hrs ($p < 0.05$). Bran did not significantly affect the number of intracellular bacteria 1 hr post-inoculation. There were significantly more viable intracellular *B. fragilis* than *B. vulgatus* 1 hr post-inoculation with or without bran ($p < 0.05$).

Table 3.3: Number of leukocytes in the murine peritoneal cavity after inoculation with abscess-inducing mixtures

Inoculum[a]	Time post-inoculation (hrs) 1.0	4.5	24
A	4.8 ± 2.6[b]	20.7 ± 4.7	12.7 ± 3.8
B	2.9 ± 1.4	11.5 ± 7.5	12.3 ± 0.4
C	4.0 ± 1.8	23.0 ± 14.8	14.1 ± 1.8
D	3.6 ± 1.8	16.1 ± 8.3	10.9 ± 0.4

a A : $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli*, 1 mg bran
B : $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli*
C : $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli*, 1 mg bran
D : $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli*

b Number of peritoneal leukocytes x 10^{-6}/mouse (mean ± 1 SD)
Represents haemocytometer counts from ≥ 3 mice

Table 3.4: Cellular content of the murine peritoneal cavity 1 hr post-inoculation with abscess-inducing mixtures

Inoculum[a]	Total Leukocytes ($x10^{-6}$)	Neutrophils ($x10^{-6}$)	% Neutrophils With Bacteria[c]	Bacteria: Neutrophils[d]	Mononuclear Phagocytes ($x10^{-6}$)	% Mononuclear Phagocytes With Bacteria[c]	Bacteria: Mononuclear Phagocytes[d]	Lymphocytes ($x10^{-6}$)	Others ($x10^{-6}$)
A	4.8 ± 2.6[b]	1.2 ± 0.7	70 ± 6	9.7 : 1	2.3 ± 1.6	13 ± 9	30.9 : 1	11.0 ± 4.0	1.3 ± 0.8
B	2.9 ± 1.4	0.66 ± 0.25	73 ± 5	12.9 : 1	1.4 ± 0.6	10 ± 7	15.3 : 1	6.8 ± 6.7	1.4 ± 0.9
C	4.0 ± 1.8	1.1 ± 0.3	75 ± 1	7.5 : 1	2.0 ± 1.1	1.0 ± 0.9	3.2 : 1	8.7 ± 5.4	0.44 ± 0.08
D	3.6 ± 1.8	1.5 ± 1.0	83 ± 11	10.7 : 1	1.4 ± 0.8	9.0 ± 3	19.0 : 1	6.6 ± 4.8	0.77 ± 0.5

a A : $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli*, 1 mg bran
B : $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli*
C : $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli*, 1 mg bran
D : $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli*

b No. peritoneal leukocytes/mouse

c % neutrophils or % mononuclear phagocytes with leukocyte-associated bacteria

d Ratio of leukocyte-associated bacteria to neutrophils or mononuclear phagocytes

Figure 3.22: Infection of the peritoneal cavity -1 hr. Peritoneal leukocytes obtained by lavage of mice inoculated 1 hr earlier with either (a) $5x10^8$ *B. fragilis*, $1x10^6$ *E. coli* and 1 mg bran, (b) $5x10^8$ *B. fragilis* and $1x10^6$ *E. coli*, (c) $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli* and 1 mg bran, or (d) $5x10^8$ *B. vulgatus* and $1x10^6$ *E. coli*.
Bacteria (arrowheads) are visible within neutrophils (pmn) and macrophages (mn).
(a), (b), (c) and (d) x 1250.

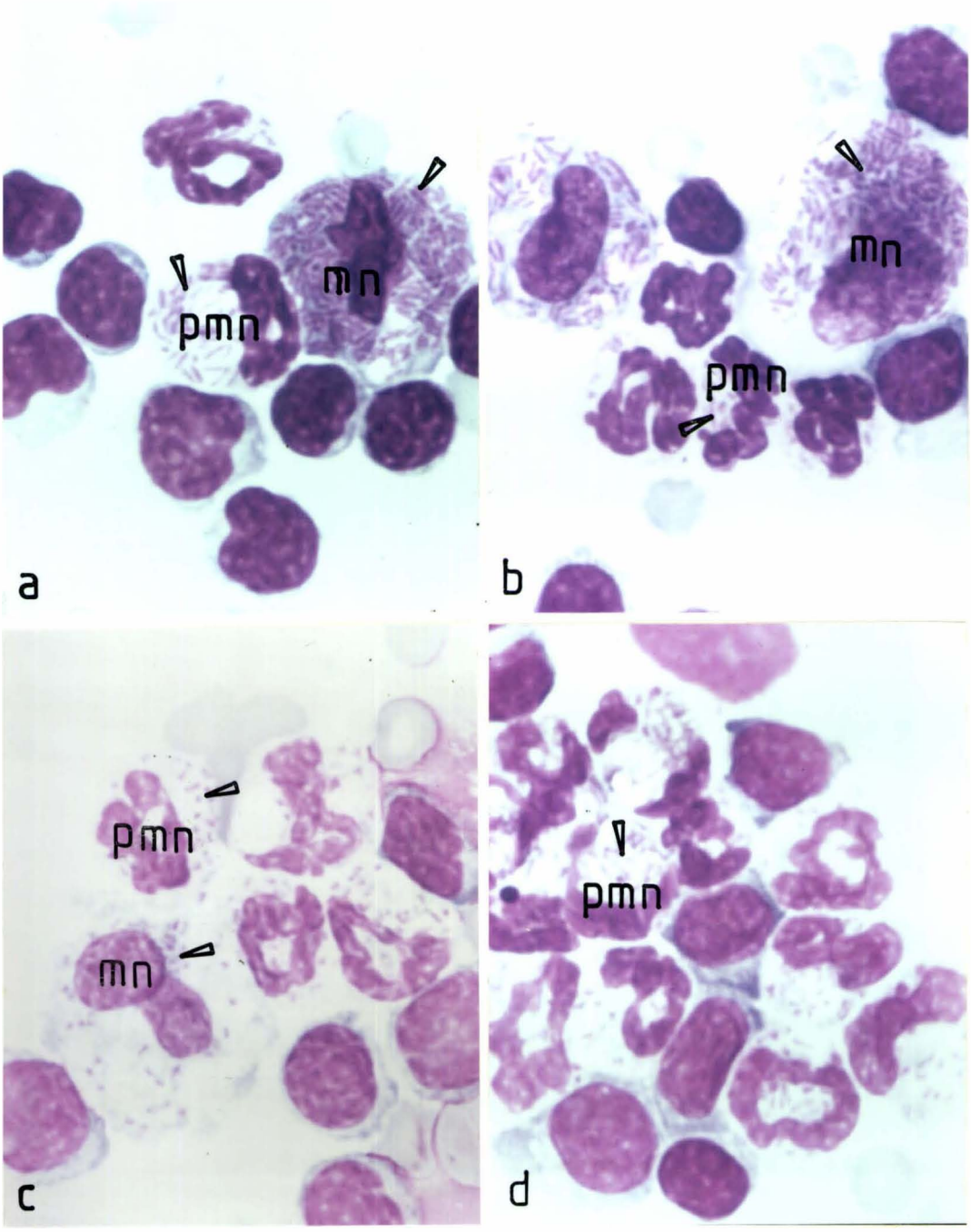

mn
pmn
a
mn
pmn
b
pmn
mn
c
pmn
d

Figure 3.23: Infection of the peritoneal cavity -4.5 hrs. Peritoneal leukocytes obtained by lavage of mice inoculated 4.5 hrs earlier with either (a) $5x10^8$ *B. fragilis*, $1x10^6$ *E. coli* and 1 mg bran, (b) $5x10^8$ *B. fragilis* and $1x10^6$ *E. coli*, (c) $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli* and 1 mg bran, or (d) $5x10^8$ *B. vulgatus* and $1x10^6$ *E. coli*. Neutrophils were the predominant cells at this time. Few intracellular bacteria were visible.
(a), (b), (c) and (d) x 1250.

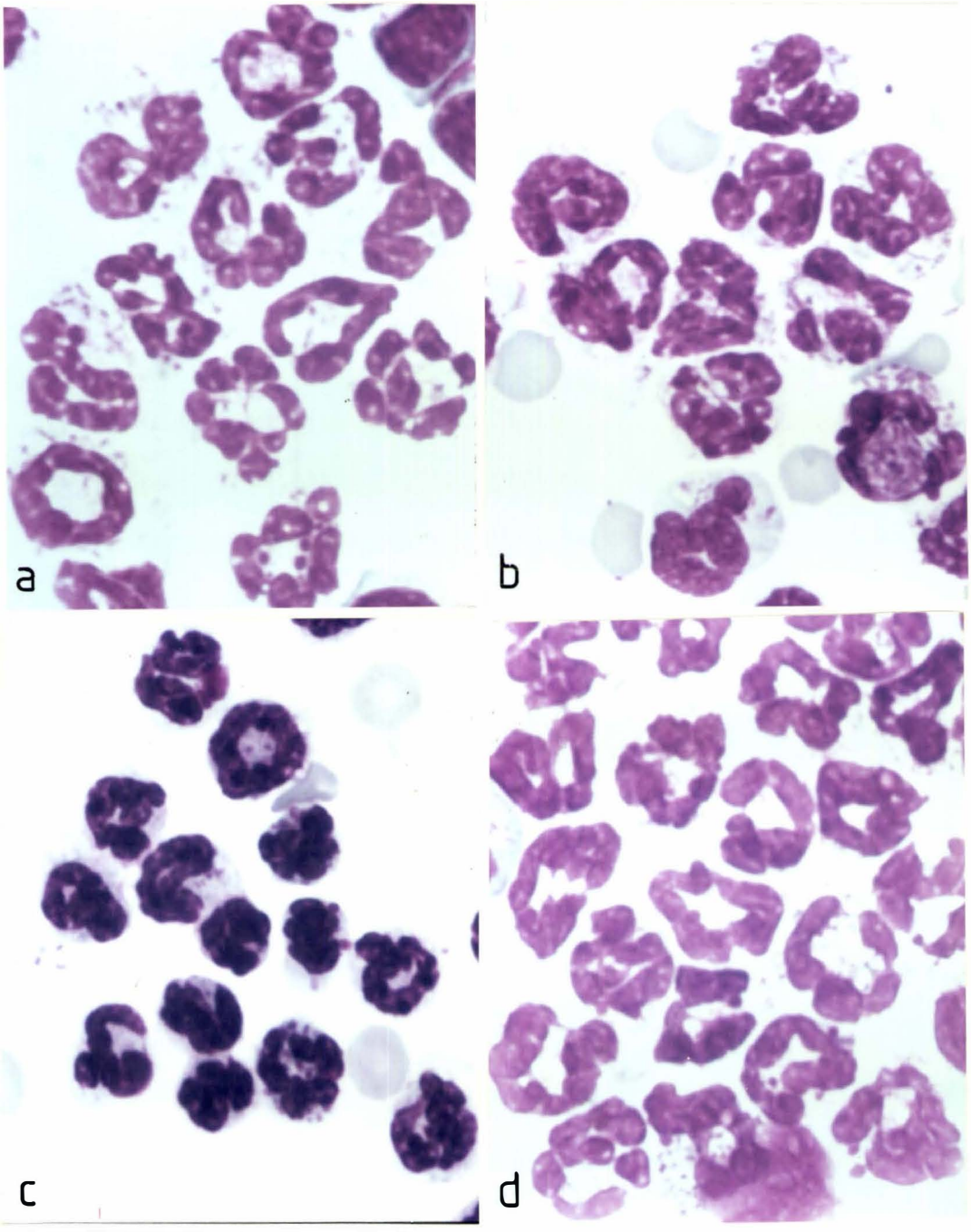
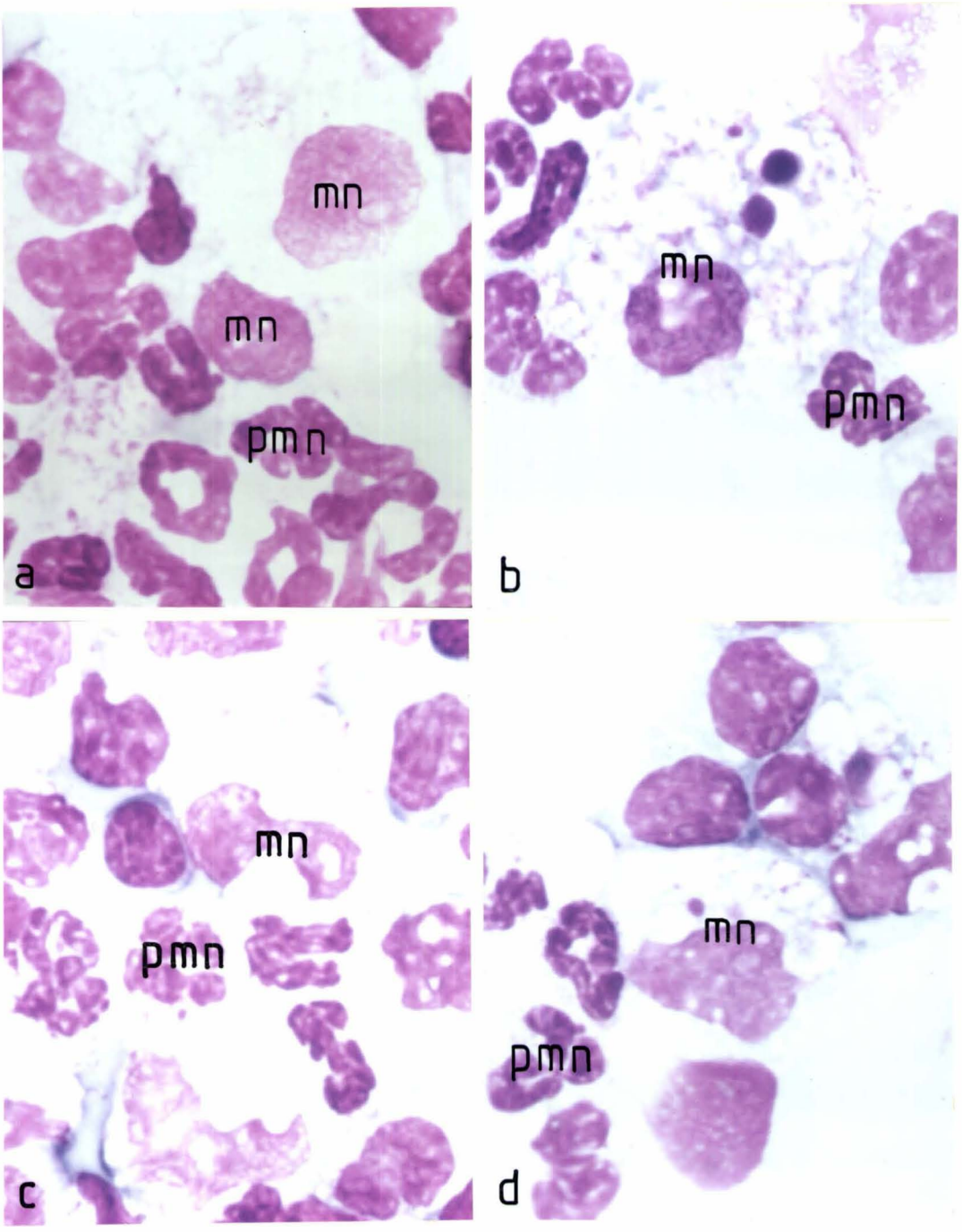

a
b
c
d

Figure 3.24: Infection of the peritoneal cavity -24 hrs. Peritoneal leukocytes obtained by lavage of mice inoculated 24 hrs earlier with either (a) $5x10^8$ *B. fragilis*, $1x10^6$ *E. coli* and 1 mg bran, (b) $5x10^8$ *B. fragilis* and $1x10^6$ *E. coli*, (c) $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli* and 1 mg bran, or (d) $5x10^8$ *B. vulgatus* and $1x10^6$ *E. coli*.
Neutrophils (pmn) and vacuolated macrophages (mn) can be seen.
(a), (b), (c) and (d) x 1250.


mn
mn
pmn
a
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b
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c
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d

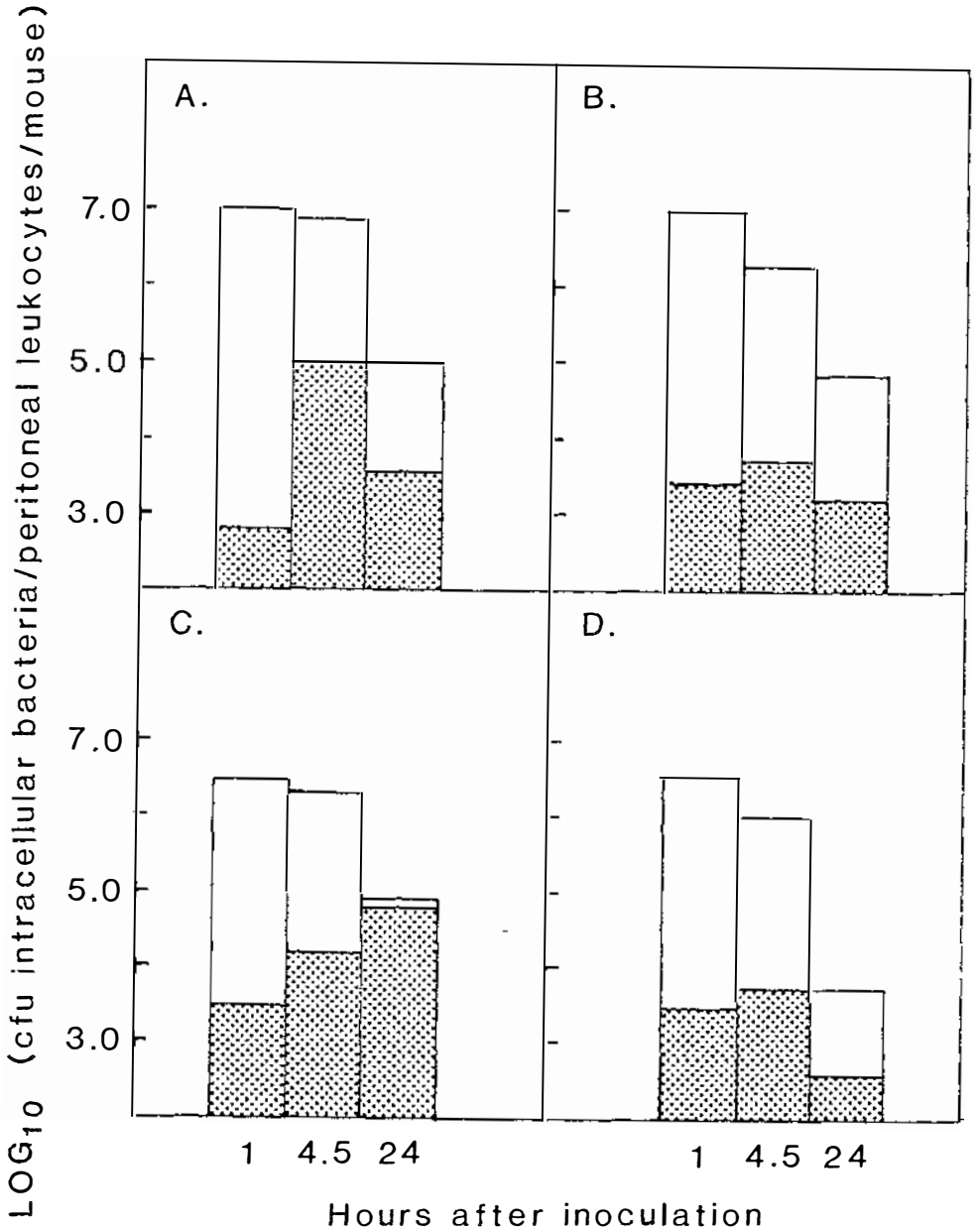


Figure 3.25: Viable intracellular bacteria after *in vivo* phagocytosis by peritoneal leukocytes. Mice were inoculated IP with either $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli* and 1 mg of bran (A), $5x10^8$ *B. fragilis* MFN 1110 and $1x10^6$ *E. coli* (B), $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli* and 1 mg of bran (C), or $5x10^8$ *B. vulgatus* and $1x10^6$ *E. coli* (D). Viable counts were performed on the washed leukocytes obtained by peritoneal lavage of 5-7 mice.

▨ = *E. coli*, □ = *Bacteroides* species

At 4.5 hrs there were signicantly more intracellular B. fragilis than B. vulgatus only if bran was present in the abscess-inducing mixture ($p < 0.05$). There were no significant differences in the numbers of E. coli within the peritoneal leukocytes obtained at 1 and 4.5 hrs following the inoculation of mice with abscess-inducing mixtures containing either B. fragilis or B. vulgatus. 24 hrs post-inoculation the numbers of B. fragilis and B. vulgatus cultured from the peritoneal leukocytes had declined by approximately 100 fold. In contrast the numbers of E. coli did not show as large a decrease at 24 hrs. In one experiment viable counts were performed on the unwashed peritoneal leukocytes to assess the total number of bacteria surviving after 24 hrs of in vivo phagocytosis. When B. fragilis, E. coli and bran were the abscess-inducing mixture, 37% of B. fragilis and 50% of E. coli were intracellular. In the absence of bran 47% of B. fragilis and 41% of E. coli were intracellular.

The ultrastructural observations of the peritoneal leukocytes during their response to the abscess-inducing mixtures are summarized in Table 3.5. When mice were inoculated IP with abscess-inducing mixtures containing bran, bacteria enmeshed in bran were occasionally seen extracellularly 1 hr post-inoculation (Fig.3.26). 1 hr post-inoculation with B. fragilis, E. coli and bran, neutrophils and macrophages were seen clumped around bran and appeared to be phagocytosing the bran (Fig.3.27a). The neutrophils and macrophages (Fig.3.27c) had many intracellular bacteria (damaged and intact) and fusion of the primary granules with some bacteria-containing phagosomes of neutrophils had occurred (Fig.3.27a & b). Neutrophils with intact peroxidase-positive and -negative granules were seen 4.5 hrs post-inoculation, along with mononuclear phagocytes (Fig.3.28a). Fewer intracellular bacteria were seen at this time (Fig.3.28b). However, 24 hrs post-inoculation with B. fragilis, E. coli and bran, more necrotic phagocytes were apparent, as indicated by the decrease in cytoplasmic electron

Table 3.5: Summary of Ultrastructural Observations on the Initiation of Abscess Development

Inoculum[a]	Hours *in vivo*[b]	Observations
A	1	Clumps of neutrophils, macrophages and bran. Mixture of damaged and intact intracellular bacteria (Fig. 3.27)
	4.5	Neutrophils and mononuclear phagocytes. Few intracellular bacteria (Fig. 3.28)
	24	Intact neutrophils and necrotic macrophages (Fig. 3.29)
B	1	Neutrophils and macrophages with a mixture of damaged and intact intracellular bacteria (Fig. 3.30)
	24	Intact neutrophils and necrotic macrophages (Fig. 3.31)
C	1	Neutrophils (Fig. 3.32) and macrophages with intracellular bacteria. Many bacteria are damaged
	4.5	Neutrophils (Fig. 3.33) and mononuclear phagocytes. Few intracellular bacteria
	24	Intact neutrophils and necrotic macrophages (Fig. 3.34)
D	1	Neutrophils and macrophages with intracellular bacteria (Fig. 3.35). Many bacteria are damaged
	24	Intact neutrophils and necrotic macrophages (Fig. 3.36)

[a] Peritoneal leukocytes from mice inoculated with:

A $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli*, 1 mg bran
B $5x10^8$ *B. fragilis* MFN 1110, $1x10^6$ *E. coli*
C $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli*, 1 mg bran
D $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli*

[b] Hours of in vivo phagocytosis before mice were sacrificed

Figure 3.26: 1 hr after the IP inoculation of $5x10^8$ B. fragilis and 1 mg of bran. Extracellular bacteria (arrowheads) are seen enmeshed in bran (Br). x 8914.

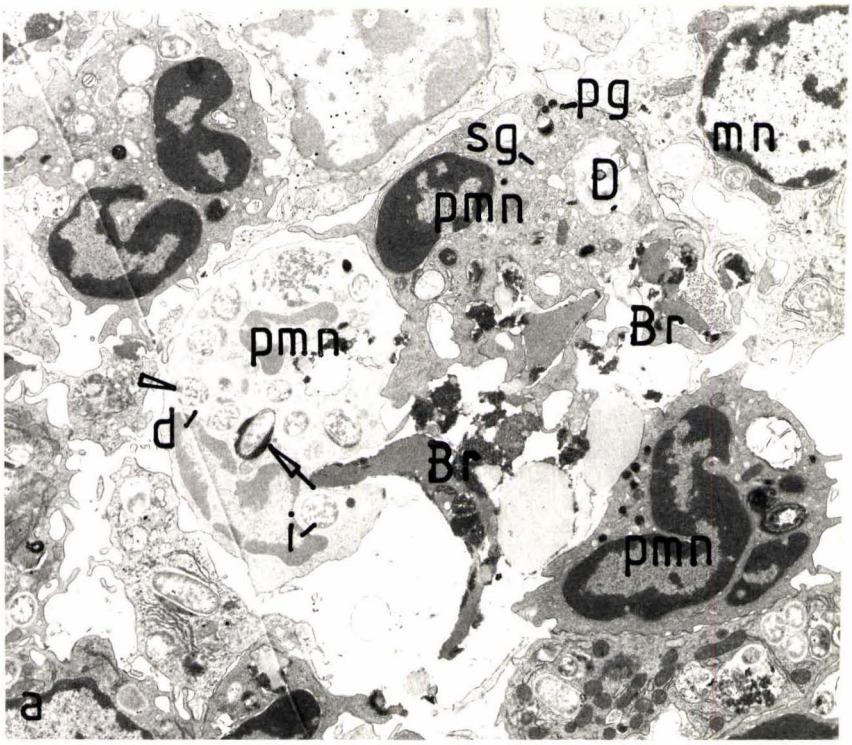
Br
Br

Figure 3.27: Intra-peritoneal inoculation of $5x10^8$ *B. fragilis*, $1x10^6$ *E. coli* and 1 mg of bran - 1 hr infection

(a) General view of neutrophils (pmn) and macrophages (mn) clumped around bran (Br). Peroxidase-positive primary granules (pg) and peroxidase-negative secondary granules (sg) are visible in the cytoplasm of some neutrophils. Bacteria are in peroxidase-negative (arrowhead) and peroxidase-positive (arrow) phagosomes, indicating phagosome-primary granule fusion has occurred. Most intra-phagosomal bacteria are intact (i), while some bacteria are damaged (d). Some phagosomes contain debris (D). x 6314.

(b) A neutrophil with peroxidase-positive primary granules (pg) and peroxidase-negative secondary granules (sg) in its cytoplasm. Damaged bacteria can be seen in peroxidase-positive phagosomes (arrows). The neutrophil also appears to be phagocytosing (arrowheads) bran (Br).
nu = neutrophil nucleus.
x 9471.

(c) An example of a macrophage which has phagocytosed many bacteria. Intact bacteria (i) and some showing initial signs of damage (d) are contained within phagosomes.
nu = macrophage nucleus.
x 9360.



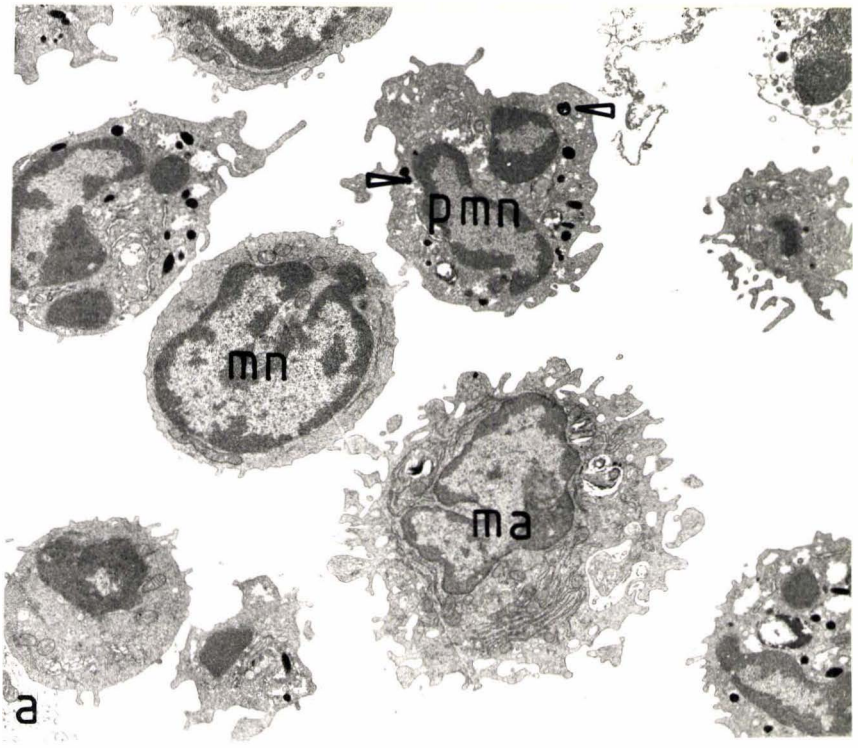
Br
nu
pg
nu
sg
nu
Br
nu
b

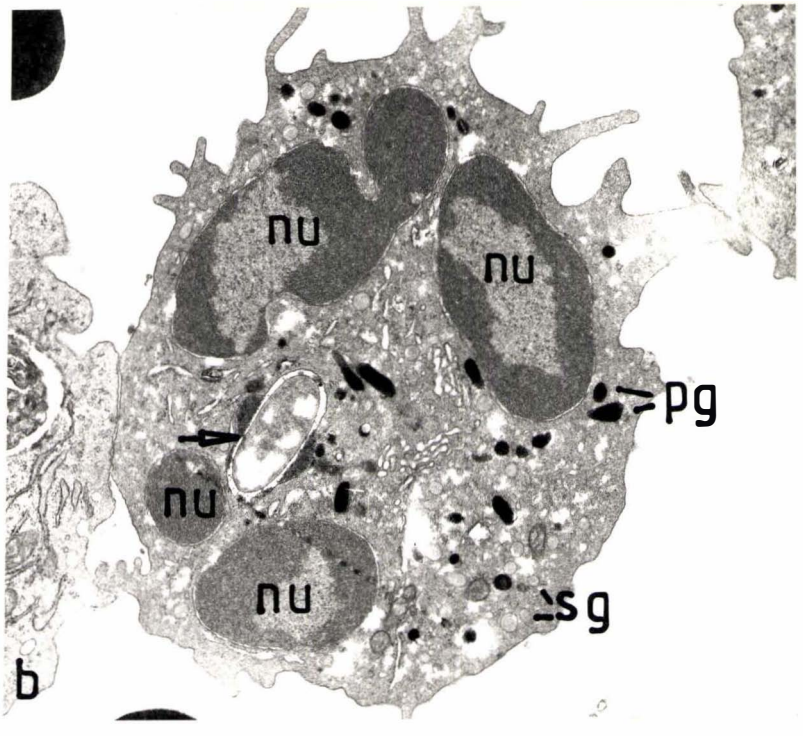
d
nu
nu
i
c

Figure 3.28: Intra-peritoneal inoculation of $5x10^8$ *B. fragilis*, $1x10^6$ *E. coli* and 1 mg of bran - 4.5 hr infection

(a) General view of neutrophils (pmn), mononuclear cells (mn) and a macrophage (ma). Peroxidase-positive primary granules (arrowheads) are visible in the cytoplasm of the neutrophils. There are no intracellular bacteria to be seen in this plane of section.
x 6314.

(b) An example of a neutrophil which has phagocytosed bacteria. Peroxidase-positive primary granules (pg) and peroxidase-negative secondary granules (sg) are in the cytoplasm. A bacterium in a peroxidase-positive phagosome (arrow) can be seen. The bacterium may be damaged, as indicated by the vacuolation of the bacterial cytoplasm.
nu = neutrophil nucleus.
x 13120.


pmn
mn
ma
a


nu
nu
pg
nu
nu
sg
b

density (Fig.3.29a). Due to the presence of a non-segmented nucleus, mitochondria and Golgi bodies, most of the necrotic cells were identified as macrophages (Fig.3.29b). Their phagosomes contained debris.

Intracellular bacteria, most of which were intact, were seen in neutrophils (Fig.3.30a) and macrophages (Fig.3.30b) 1 hr post-inoculation with B. fragilis and E. coli alone. Fig.3.31 shows a neutrophil and a necrotic macrophage 24 hrs post-inoculation.

Damaged bacteria were seen in phagosomes of neutrophils 1 hr following the IP inoculation of B. vulgatus, E. coli and bran (Fig.3.32). Bran can be seen in one bacteria-containing phagosome of the neutrophil. Fig.3.33 shows neutrophils and extracellular bran 4.5 hrs post-inoculation. Damaged bacteria are visible in phagosomes of one neutrophil. Necrotic macrophages and intact neutrophils were seen 24 hrs post-inoculation (Fig.3.34a). The necrotic macrophages contained debris within their phagosomes (Fig.3.34b).

Mixtures of damaged and intact bacteria were seen in phagosomes of neutrophils 1 hr following the inoculation of B. vulgatus and E. coli alone (Fig.3.35a). Fig.3.35b shows a neutrophil with damaged bacteria in peroxidase-positive phagosomes, indicating fusion of some primary granules has occurred. Peroxidase-negative granules are visible in the neutrophil's cytoplasm. Macrophages had also phagocytosed bacteria and by 1 hr post-inoculation, some bacteria were damaged (Fig.3.35c). Fig.3.36 shows a necrotic macrophage 24 hrs post-inoculation with B. vulgatus and E. coli. The phagosomes of the macrophage contain debris.

Peritoneal leukocytes which had phagocytosed E. coli and either B. fragilis or B. vulgatus in the presence and absence of bran in vivo for 1 or 24 hrs were incubated in vitro with 10% NS and the killing of the ingested bacteria was monitored (Table 3.6).

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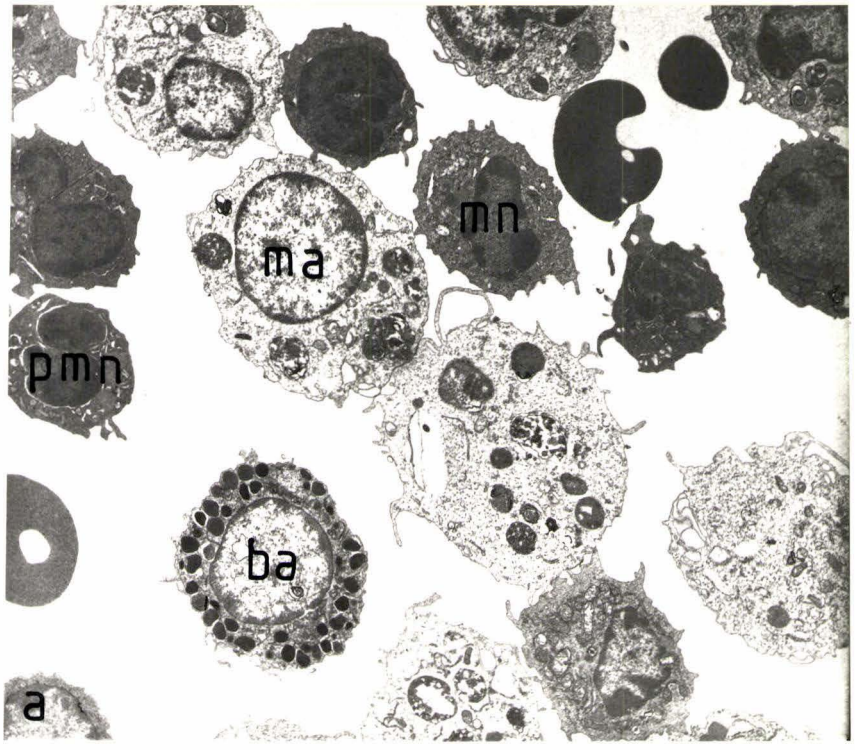
Figure 3.29: Intra-peritoneal inoculation of $5x10^8$ *B. fragilis*, $1x10^6$ *E. coli* and 1 mg of bran - 24 hr infection

(a) General view of intact neutrophils (pmn), mononuclear cells (mn) and necrotic macrophages (ma). ba = basophil. x 4129.

(b) An example of a necrotic macrophage. The cytoplasm of the cell has a reduced electron density and contains mitochondria(m), a Golgi body (go) and phagosomes (p) containing debris. nu = macrophage nucleus. x 8914.


mn
ma
pmn
ba
a

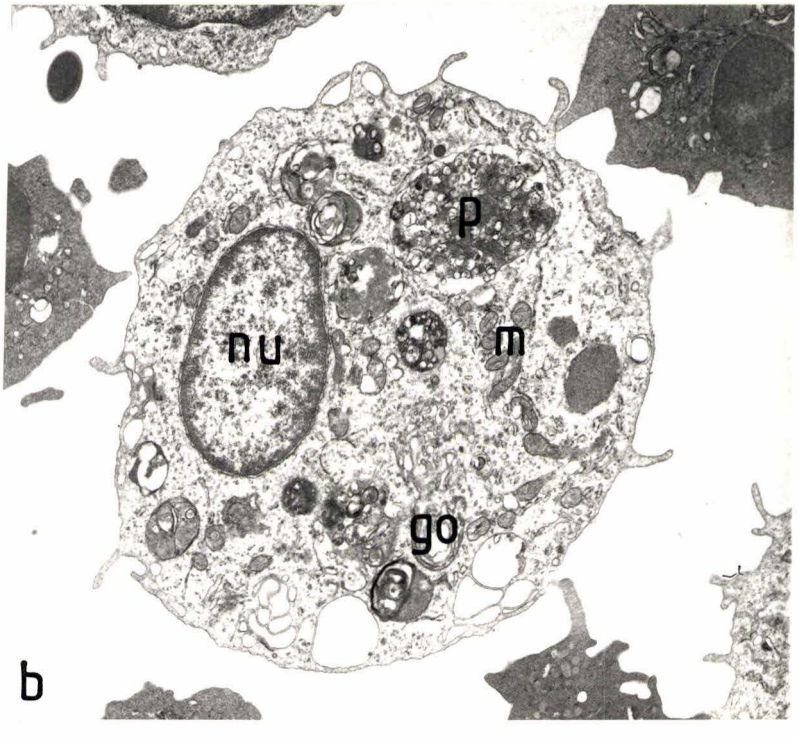
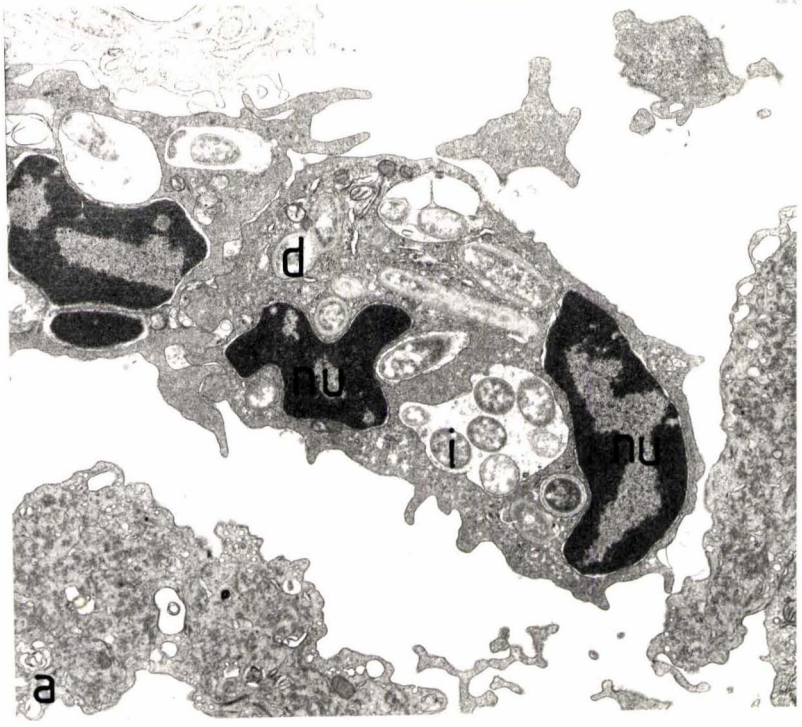
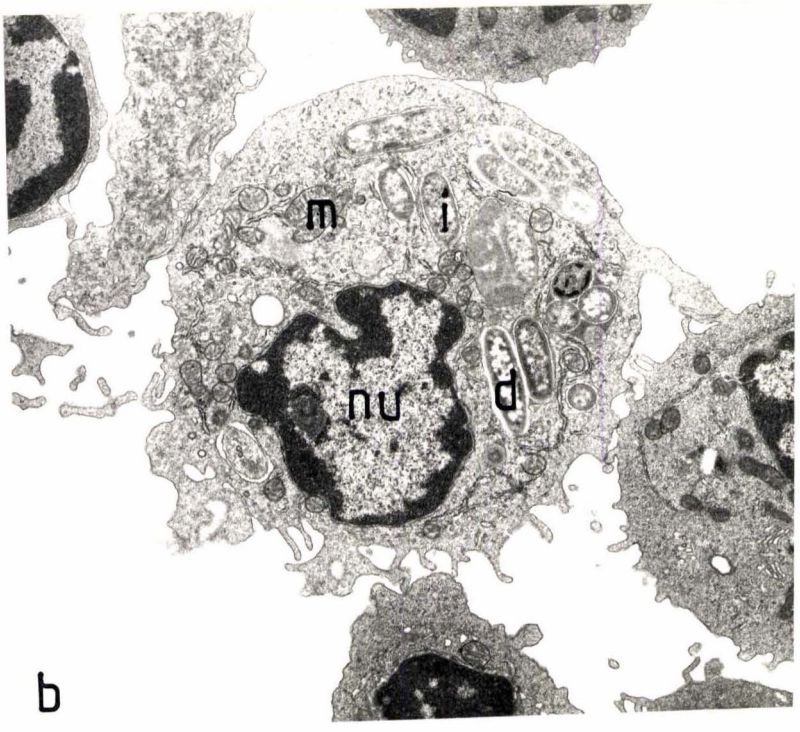

p
nu
m
go
b

Figure 3.30: Intra-peritoneal inoculation of $5x10^8$ *B. fragilis* and $1x10^6$ *E. coli* - 1 hr infection

(a) A neutrophil containing intact (i) and damaged (d) bacteria in peroxidase-negative phagosomes.
nu = neutrophil nucleus.
x 9360.

(b) A macrophage containing intact (i) and damaged (d) bacteria in phagosomes.
nu = macrophage nucleus,
m = mitochondria.
x 9360.


d
nu
i
nu
a


m
i
nu
d
b

Facing Page 104

Figure 3.31: Intra-peritoneal inoculation of $5x10^8$ *B. fragilis* and $1x10^6$ *E. coli* - 24 hr infection

A neutrophil (pmn) and a necrotic macrophage (ma). The macrophage contains phagosomes (arrowheads) filled with debris.
x 6314.

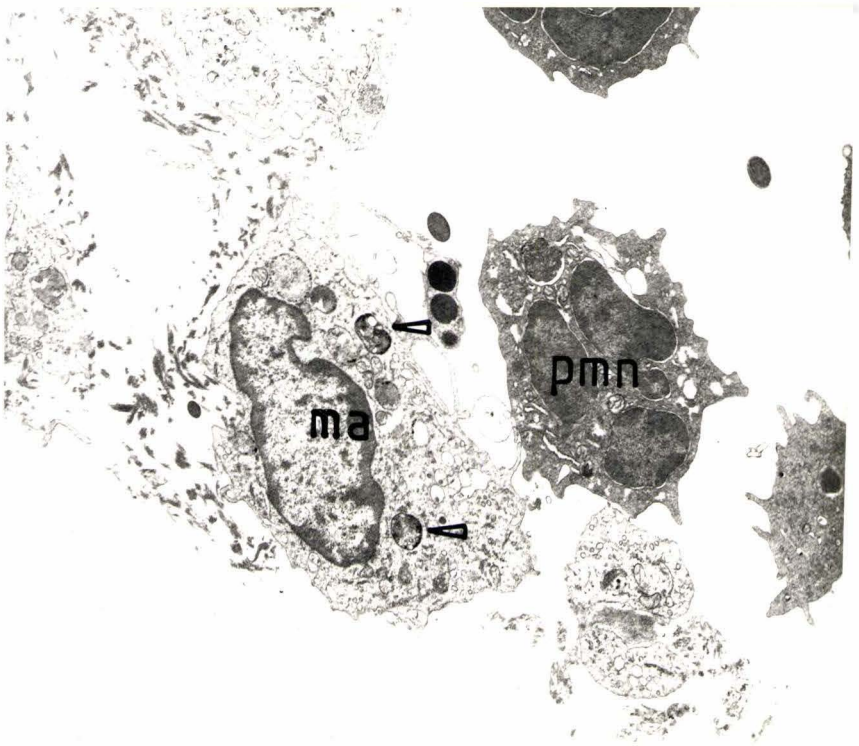

pmn
ma

Figure 3.32: Intra-peritoneal inoculation of $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli* and 1 mg of bran - 1 hr infection

A neutrophil containing bacteria (b) displaying varying degrees of damage in peroxidase-negative (arrowheads) and peroxidase-positive (arrow) phagosomes. A peroxidase-positive primary granule (pg) is close to a peroxidase-negative phagosome. Peroxidase-negative secondary granules (sg) are visible in the cytoplasm. Bran (Br) can be seen in the largest phagosome.
nu = neutrophil nucleus.
x 13600.

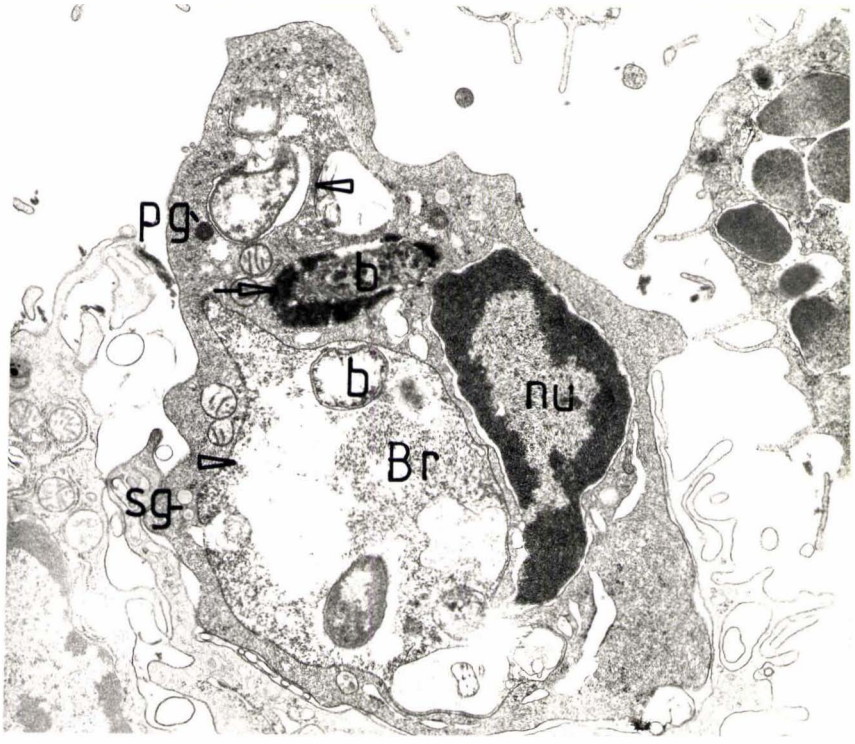

pg
b
b
nu
Br
sg

Figure 3.33: Intra-peritoneal inoculation of $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli* and 1 mg of bran - 4.5 hr infection

Neutrophils (pmn) and extracellular bran (Br). Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules can be seen in one neutrophil. Damaged bacteria (arrowheads) are visible in phagosomes of the other neutrophil.
x 6686.

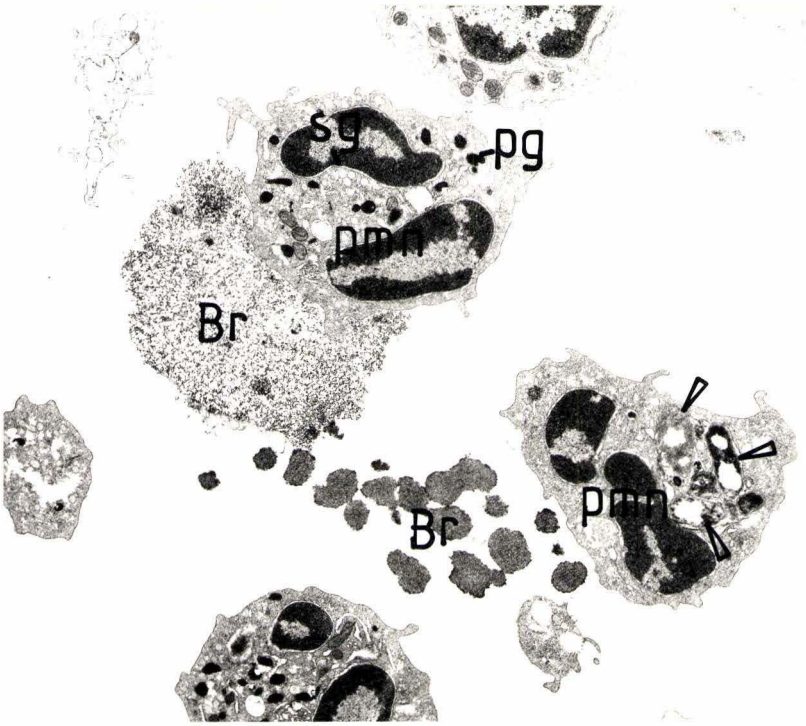
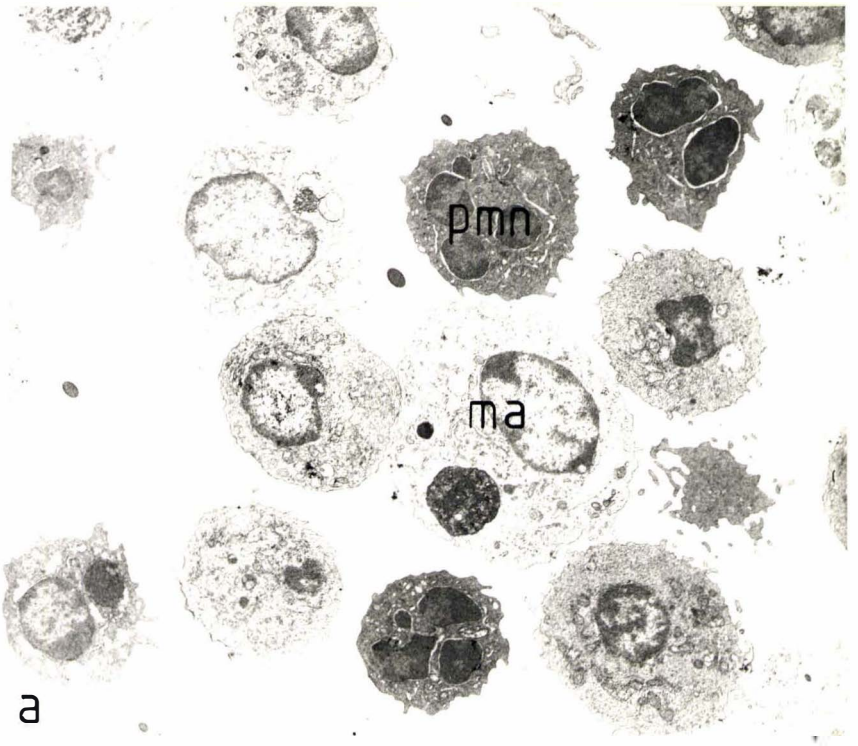

sg
pg
pmn
Br
Br
pmn

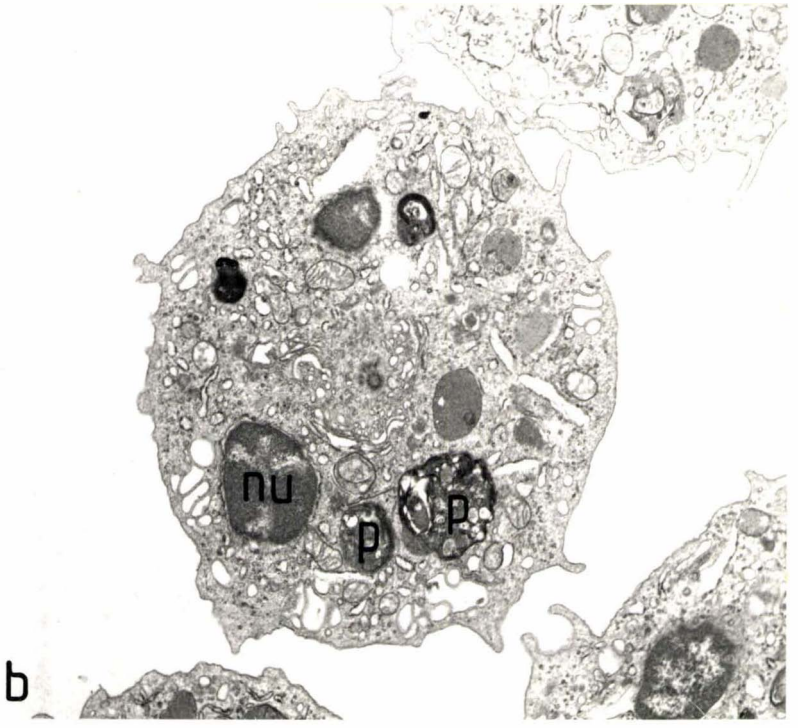
Figure 3.34: Intra-peritoneal inoculation of $5x10^8$ *B. vulgatus*, $1x10^6$ *E. coli* and 1 mg of bran - 24 hr infection

(a) General view of intact neutrophils (pmn) and necrotic macrophages (ma). x 4129.

(b) A necrotic macrophage showing the phagosomes (p) filled with debris. nu = macrophage nucleus. x 9806.



a



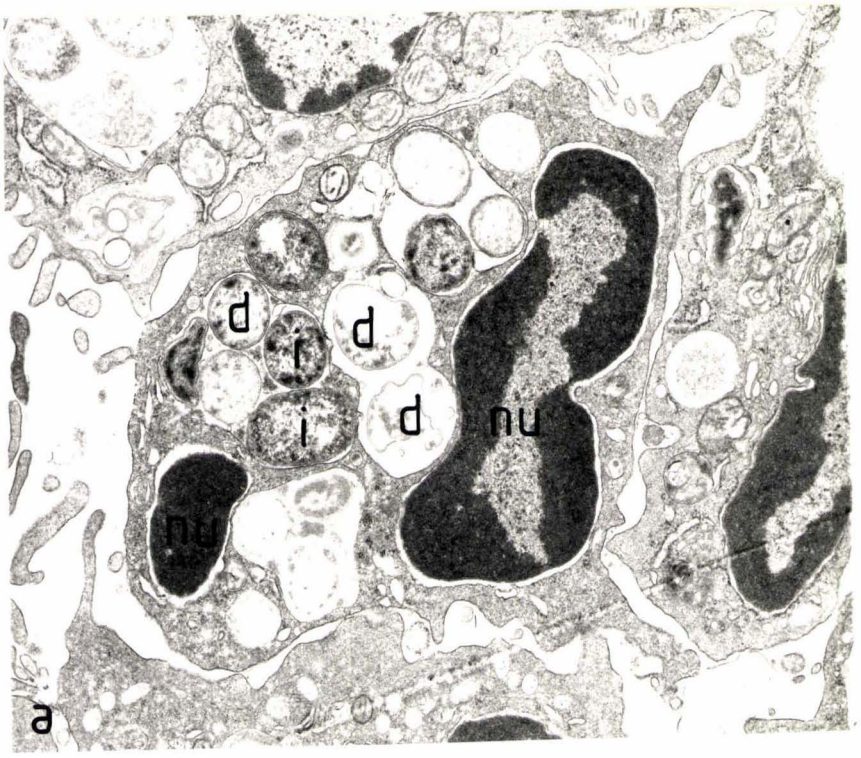
b

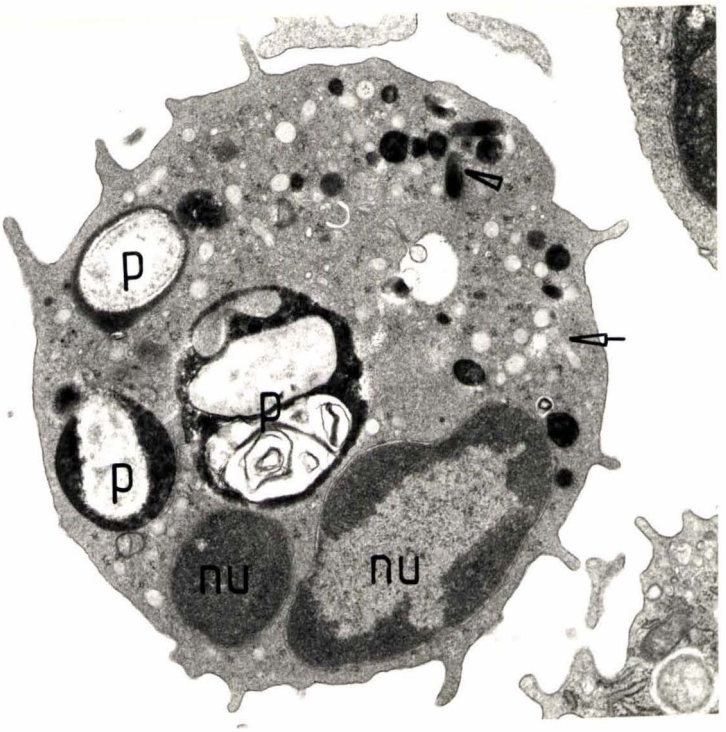
Figure 3.35: Intra-peritoneal inoculation of $5x10^8$ B. vulgatus and $1x10^6$ E. coli - 1 hr infection

(a) Neutrophil with phagosomes containing damaged bacteria (d) in a variety of states, plus some intact bacteria (i).
nu = neutrophil nucleus.
x 13600.

(b) Neutrophil with peroxidase-positive primary (arrowhead) and peroxidase-negative secondary (arrow) granules in its cytoplasm. The peroxidase-positive phagosomes (p) contain damaged bacteria.
nu = neutrophil nucleus.
x 15040.

(c) Macrophage containing damaged (d) and intact (i) bacteria in phagosomes.
nu = macrophage nucleus.
x 8914.


d
d
i
d
nu
nu
a


p
p
p
nu
nu
b

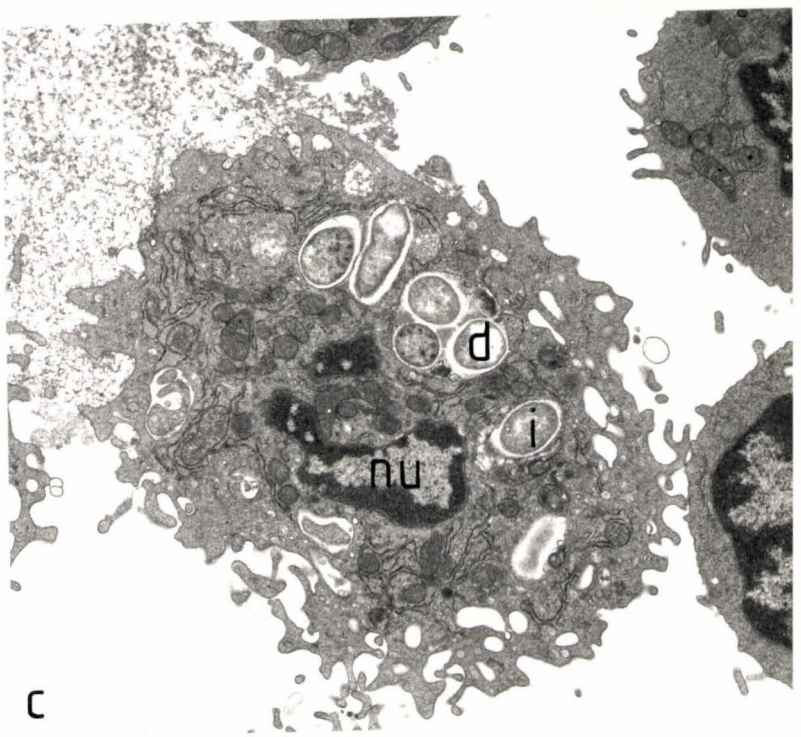

d
i
nu
c

Figure 3.36: Intra-peritoneal inoculation of $5x10^8$ *B. vulgatus* and $1x10^6$ *E. coli* - 24 hr infection.

An example of a necrotic macrophage containing phagosomes (p) filled with debris.
nu = macrophage nucleus.
x 6314.

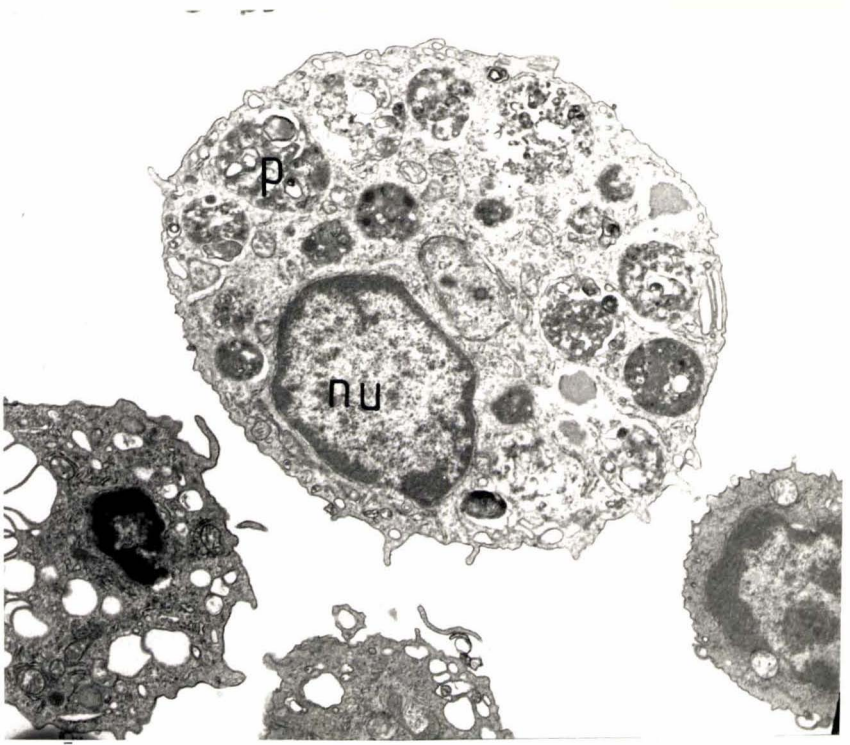

P.
nu

Table 3.6: Killing *in vitro* of Bacteria Phagocytosed *in vivo*

Leukocytes[a]	Hours *in vivo*[b]	Peritoneal leukocytes: bacteria[c] Bacteroides	E. coli	ΔB[d] Bacteroides	E. coli
A	1	1 : 2.3	7.5×10^3:1	- 0.17	+ 1.42
	24	135.1 : 1	3.6×10^3:1	- 0.01	+ 0.25
B	1	1 : 3.4	1.2×10^3:1	- 0.23	+ 0.41
	24	159.7 : 1	7.7×10^3:1	- 0.04	+ 0.29
C	1	1.2 : 1	1.3×10^3:1	- 0.39	+ 0.93
	24	172.0 : 1	235.0 :1	- 0.53	+ 0.19
D	1	1.2 : 1	1.3×10^3:1	- 0.20	+ 0.68
	24	2.3×10^3:1	2.9×10^4:1	- 0.68	- 0.05

[a] Peritoneal leukocytes from mice inoculated with

A 5×10^8 *B. fragilis* MFN 1110, 1×10^6 *E. coli*, 1 mg bran
B 5×10^8 *B. fragilis* MFN 1110, 1×10^6 *E. coli*
C 5×10^8 *B. vulgatus*, 1×10^6 *E. coli*, 1 mg bran
D 5×10^8 *B. vulgatus*, 1×10^6 *E. coli*

[b] Hours of *in vivo* phagocytosis before mice were sacrificed

[c] Ratio of peritoneal leukocytes to intracellular bacteria at the start of the *in vitro* assays

[d] $\log_{10}$ change in viable count after 2 hrs aerobic incubation in 10% NS. Mean of 2 expts

At the start of the *in vitro* assays, after 1 hr of phagocytosis *in vivo*, the ratios of peritoneal leukocytes to *B. vulgatus* and *B. fragilis* ranged from 1:1 to 1:3 respectively. However, after 24 hrs of phagocytosis *in vivo*, the ratios of peritoneal leukocytes to *B. fragilis* and *B. vulgatus* ranged from 135:1 to 2.3×10^3:1. After either 1 or 24 hrs of phagocytosis *in vivo* there were high ratios of peritoneal leukocytes to *E. coli*.

When peritoneal leukocytes which had phagocytosed *B. fragilis* or *B. vulgatus* in the presence of bran and *E. coli* *in vivo* for 1 hr were incubated *in vitro* with 10% NS, there was less killing of *B. fragilis* than of *B. vulgatus* after 120 mins. After 24 hrs *in vivo* phagocytosis in the presence of bran and *E. coli*, *in vitro* killing of intracellular *B. fragilis* in NS was negligible, whereas 72% of intracellular *B. vulgatus* had been killed by 120 mins. When *B. fragilis* and *B. vulgatus* were phagocytosed *in vivo* in the absence of bran, differences in their *in vitro* killing in NS were apparent only after 24 hrs of *in vivo* phagocytosis. Again, killing of intracellular *B. fragilis* was slight, whereas 78% of intracellular *B. vulgatus* had been killed after 120 mins incubation *in vitro*. There was no overall *in vitro* killing of *E. coli* which had been phagocytosed *in vivo* except after 24 hrs *in vivo* phagocytosis with *B. vulgatus* in the absence of bran. However, there were reductions in the log increase of the number of *E. coli* cultured *in vitro* at 120 mins after 24 hrs of phagocytosis compared with 1 hr of phagocytosis *in vivo* (Table 3.6).

3.3 DISCUSSION

3.3.1 Abscess Development: B. fragilis, E. coli and Bran

A model of IA abscess formation in Balb/c mice (Nulsen et al., 1983), which uses total abscess weight per mouse and the bacterial content of abscesses to quantify abscess development, was successfully used in C3H mice (Table 3.1, Fig.3.2). B. fragilis and E. coli, which are components of the abscess-inducing mixture, are frequently isolated together from human cases of IA sepsis (Bartlett, 1981). However, IA sepsis in humans due to the contamination of the peritoneal cavity with intestinal contents may not be solely due to the bacteria. The complex aetiology is reflected in the need for potentiating agents, such as autoclaved intestinal contents, in animal models of IA sepsis (Onderdonk et al., 1976; Joiner et al., 1980b; Nulsen et al., 1983). Bran was used as the potentiating agent in this study and was necessary for the induction of persistent abscesses (Table 3.1, Fig.3.2 cf. Table 3.2, Fig.3.3).

Abscesses induced by AIM persisted for at least ten weeks in the peritoneal cavities of C3H mice as they did in Balb/c mice. However, from six days post-inoculation onwards, IA abscesses in C3H mice were larger and contained more viable bacteria than those from Balb/c mice, suggesting strain differences exist in the host immune defences. Abscess development within C3H mice inoculated IP with AIM was characterized by an increase in the weight of abscesses per mouse over a ten week period (Table 3.1). At ten weeks abscesses were found only in the groin area or attached to the mesentery and intestines.

Viable B. fragilis and E. coli persisted in abscesses induced by AIM for the ten week period under study (Fig.3.2). During this time it is likely that some bacteria will have died or

have been killed by phagocytes. However, at ten weeks the numbers of viable B. fragilis and E. coli were similar to those in AIM, which suggests that bacterial replication can occur within the abscesses.

Histologically, the IA abscesses in C3H mice were similar to those in Balb/c mice (Nulsen et al., 1983), except in C3H murine abscesses the central necrotic area of neutrophils was extensive in older abscesses and mononuclear phagocytes did not appear to accumulate to the same extent as abscesses aged (Fig.3.6). The evacuation through the skin of C3H mice of some older abscesses, which had attached to the peritoneum, was probably due to the high osmotic pressure of pus. The pressure within an abscess can cause the abscess to move along the line of least resistance and evacuate through the skin (Murphy, 1976).

The distribution of B. fragilis antigens in abscesses induced in C3H mice (Fig.3.7) was similar to that in abscesses induced in Balb/c mice (Nulsen et al., 1983). However, E. coli antigens were found only in the central necrotic area of abscesses in C3H mice and were not detected in the area of mononuclear phagocytes as in abscesses from Balb/c mice. These histological differences between abscesses from C3H and Balb/c mice are consistent with the strain differences observed in relation to the weight and bacterial content of abscesses, as discussed earlier.

The observation that neutrophils were the predominant cells in histological sections of IA abscesses induced by B. fragilis, E. coli and bran is consistent with the description of staphylococcal IA abscesses in mice (Kapral et al., 1980), whereas abscesses induced by B. fragilis and E. coli in the absence of bran did not have a similar cellular organization due to the more rapid resolution of the abscesses (Section 3.2.3). The ultrastructure of murine peritoneal neutrophils

(Section 3.2.4), including the diverse morphology of the cytoplasmic granules, is similar to that of human (Hirsch & Fedorko, 1968) and rabbit (Bainton & Farquhar, 1966) neutrophils.

Neutrophil primary, or azurophilic, granules contain the microbicidal enzyme myeloperoxidase (Root & Cohen, 1981) which has been used as a marker of the granules in EM studies of neutrophil development (Bainton & Farquhar, 1966) and phagosome-lysosome fusion within neutrophils (Bainton, 1973). In the present study, labelling of murine peritoneal neutrophils for peroxidase activity revealed the peroxidase-positive primary granules were outnumbered by the peroxidase-negative granules (Figs.3.9 and 3.10) which has been demonstrated for rabbit (Bainton & Farquhar, 1966) and human (Scott & Horn, 1970; Bainton et al., 1971) neutrophils. Mature neutrophils from murine bone marrow have been shown to have a mean number of 33 primary granules and 148 secondary granules per neutrophil (Ogawa et al., 1983).

The enzyme alkaline phosphatase has been used in EM studies as a marker for neutrophil secondary granules (Bainton, 1973) but some animal species, e.g. mice, lack this enzyme within their neutrophils (Rausch & Moore, 1975). Often secondary granules are identified simply by the absence of peroxidase staining. However, secondary granules of guinea pig (Noseworthy et al., 1975), rabbit (Parmley et al., 1980) and human (Fittschen et al., 1983) neutrophils have been shown to contain large amounts of glycoprotein. Fittschen et al. (1983), in an ultrastructural study of the development of neutrophil secondary granules, were able to demonstrate that the PA-TCH-SP reaction stained the glycoproteins of the secondary granules. In the present study PA-TCH-SP staining of murine peritoneal neutrophils did not clearly separate the secondary granules from the primary granules (Fig.3.13). This may have been due to granules containing varying

quantities of glycoprotein which would influence the intensity of the staining. Indeed, Fittschen et al. (1983) found that some primary granules of mature human neutrophils were stained weakly by the PA-TCH-SP method. Recently a method combining peroxidase labelling and PA-TCH-SP staining was used on feline neutrophils (Fittschen et al., 1988). Peroxidase-positive granules, PA-TCH-SP positive granules and a third type of granule which was both peroxidase- and PA-TCH-SP-positive were identified. The possibility that the third type of granule was a primary granule which contained some glycoprotein was not discussed by the authors.

Neutrophils are responsible for much of the liquefactive necrosis which results in abscess development and continues within the abscess. This is due to the secretion of tissue-damaging and microbicidal substances from neutrophil granules either actively, upon phagocytosis or due to death of the neutrophil (Wright, 1982). Thus, the pus of the abscess, which is contained by granulation tissue, is composed of live and intact, degenerating and dead neutrophils, viable and non-viable bacteria and debris from dead host cells and bacteria suspended in inflammatory exudate (Hurley, 1983). The histology of IA abscesses induced by AIM in C3H mice (Section 3.2.3) was consistent with this description of suppuration followed by abscess development.

The ultrastructural observations on the development of IA abscesses induced by AIM (Section 3.2.5) also confirmed the interpretation of the IA abscess histology (Section 3.2.3). Hurley (1983) states that the replacement of neutrophils by macrophages does not occur in suppurative inflammation and mononuclear phagocytes were rarely seen in IA abscesses. Indeed, neutrophils emigrated from the blood vessels of the granulation tissue into the interior of the abscess (Fig.3.17c) as described by Hurley (1983). Intracellular (Figs.3.15 and 3.16b) and extracellular (Figs.3.17a, 3.18b,

3.19a and 3.20) bacteria were seen in the interior of IA abscesses, which was consistent with the distribution of bacterial antigens in histological sections of abscesses (Fig.3.7).

The lifespan of a neutrophil in sites of inflammation may range from a few hours (Hurley, 1983) to 3-4 days (Murphy, 1976) and it is possible some extracellular bacteria within the abscesses may have survived the intracellular environment of the neutrophil and been released upon the death of the phagocyte. Neutrophil-associated and extracellular Neisseria gonorrhoeae are seen in urethral pus and Veale et al. (1977) argue that due to the large number of neutrophils, it is unlikely the extracellular bacteria have never been phagocytosed. The same argument could be applied to extracellular bacteria seen in IA abscesses. Viable bacteria were present in IA abscesses up to ten weeks old (Fig.3.2), which indicates some resistance to the microbicidal mechanisms of neutrophils.

In summary, a model of IA abscess formation developed in Balb/c mice was used in C3H mice. In-depth histological and ultrastructural studies confirmed that the neutrophil was the predominant cell in the abscesses. Studies of abscess development and histology showed strain differences existed which probably reflect differences in the host immune defences, such as complement activity. The amount of complement activity in various strains of mice has been shown to differ (Terry et al., 1963). Fresh NS from C3H mice did not support in vitro killing of E. coli by peritoneal neutrophils from C3H or Balb/c mice to the same extent as fresh Balb/c NS (Finlay-Jones, personal communication). Thus, in the peritoneal cavity of the non-immune C3H mouse, if the complement system is the main source of opsonins, the ability of neutrophils to kill bacteria may be compromised. The protracted survival of many bacteria would attract more

neutrophils to the site, which may ultimately result in the formation of larger abscesses. Despite the phagocytic ability of the neutrophil, chronic reservoirs of viable B. fragilis and E. coli, some of which were extracellular, remained within the abscesses. Thus, although the IA abscesses serve to contain the pathogens, in the long term they fail as a means of eliminating the bacteria in this murine model.

3.3.2 Abscess Development: Other Abscess-Inducing Mixtures

B. fragilis ATCC 23745 has been extensively studied in a rat model of IA sepsis (Onderdonk et al, 1984). In the presence of potentiating agents the encapsulated B. fragilis ATCC 23745 could induce abscesses to form either alone or in combination with a facultative anaerobe. In the present study B. fragilis ATCC 23745, which had been passaged five times in murine IA abscesses, was shown to be encapsulated (Fig.3.1d). In the presence of bran, B. fragilis ATCC 23745, either alone or combined with E. coli, induced abscesses to form (Table 3.2).

Abscesses induced by encapsulated B. fragilis ATCC 23745, E. coli and bran were significantly smaller than those induced by AIM and in comparison with encapsulated B. fragilis MFN 1110 (Fig.3.1a and b), fewer viable B. fragilis ATCC 23745 survived within the abscesses three weeks post-inoculation (Fig 3.2 cf. Fig.3.3). Synergistic relationships between anaerobic and facultatively anaerobic species of IA abscess-inducing bacteria, especially B. fragilis and E. coli, have been demonstrated and many mechanisms of synergy suggested (Rotstein et al., 1985c). The differences observed in this study between abscesses induced by mixtures containing either B. fragilis MFN 1110 or B. fragilis ATCC 23745 may be due to differences in the relationship between each of these strains

and E. coli. Verweij-van Vught et al. (1985) demonstrated synergy between one pair of B. fragilis and E. coli strains but not between another pair of strains in a murine model of SC infection. Synergy was interpreted as present if B. fragilis reduced the clearance of E. coli from the site of infection.

Recent studies have compared the pathogenicity of B. fragilis and B. vulgatus in mixed infections (Vel et al., 1986; Verweij-van Vught et al., 1986). B. vulgatus outnumbers B. fragilis in the human colon, yet it is infrequently isolated from mixed IA infections (Bartlett, 1981). In the present study, encapsulated B. vulgatus (Fig.3.1e) induced the formation of smaller IA abscesses than did encapsulated B. fragilis MFN 1110 and the abscesses contained fewer viable B. vulgatus (Table 3.1, Fig.3.2 cf. Table 3.2, Fig.3.3). By six days post-inoculation there had been a large decrease in the number of viable B. vulgatus within the abscesses. Verweij-van Vught et al. (1986) demonstrated that when single strains were inoculated, B. vulgatus was more rapidly cleared from the SC tissue of mice than was B. fragilis. They concluded that the ability of B. fragilis and B. vulgatus strains to induce abscess formation, in combination with E. coli, depended not only on the synergy between the Bacteroides species and E. coli, but also on the inherent virulence of the anaerobe.

Thus, when IA abscess development was studied in C3H mice (Tables 3.1 and 3.2, Figs.3.2 and 3.3), it appeared that B. fragilis MFN 1110 was more virulent than B. fragilis ATCC 23745, which in turn was more virulent than the B. vulgatus strain when combined with E. coli and bran. Therefore, in further in vivo (Section 3.2.6) and in vitro (Sections 4.2.2, 4.2.3 and 4.2.5) experiments, the virulent B. fragilis MFN 1110 was compared with the least virulent organism, B. vulgatus.

3.3.3 Initiation of Abscess Development

The host defence mechanisms which exist to control the infection of the peritoneal cavity are phagocytosis, clearance of phagocytosed and non-phagocytosed bacteria via diaphragmatic lymphatic absorption, plus trapping of bacteria in fibrin clots (Dunn et al., 1985b). The phagocytic responses to infection of the murine peritoneal cavity with abscess-inducing mixtures, containing either B. fragilis or B. vulgatus, were compared in the present study (Section 3.2.6). After IP inoculation of mice with abscess-inducing mixtures, lavage readily provided a population of leukocytes that had phagocytosed bacteria. Light and electron microscopy were used to examine the phagocytosed bacteria, but since the abscess-inducing mixtures contained 500-fold fewer E. coli than Bacteroides species, most of the bacteria observed were assumed to be Bacteroides species.

The development of IA abscesses begins with an acute inflammatory response in which the neutrophil is the predominant cell. Thus, 1 hr post-inoculation with abscess-inducing mixtures containing either B. fragilis or B. vulgatus, more neutrophils had phagocytosed bacteria than had mononuclear phagocytes (Table 3.4, Fig.3.22). Macrophages are considered to be more important in dealing with facultative or obligate intracellular bacteria (Elsbach, 1980). Furthermore, encapsulated B. fragilis (Rodloff et al., 1986) and the endotoxin of E. coli (Hammerström & Unsgaard, 1979; Vogel et al., 1979) have been reported to inhibit macrophage phagocytic activity.

The ratios of total leukocyte-associated bacteria (viable and non-viable) to neutrophils 1 hr post-inoculation with the four different abscess-inducing mixtures were similar,

suggesting the susceptibility of B. fragilis and B. vulgatus to phagocytosis did not differ between groups (Table 3.4). However, there were significantly fewer viable intracellular B. vulgatus than B. fragilis at this time (Fig.3.25), implying more B. vulgatus had been killed by the leukocytes. This was generally consistent with the in vitro killing of bacteria which had been phagocytosed in vivo for 1 hr (Table 3.6). Furthermore, damaged intracellular B. vulgatus (Figs.3.32 and 3.35) were more frequently seen than were damaged intracellular B. fragilis (Figs.3.27 and 3.30) 1 hr post-inoculation (Table 3.5).

4.5 hrs after inoculation there had been an increase in the number of peritoneal leukocytes (Table 3.3), predominantly due to the influx of more neutrophils (Fig.3.23) which had been involved in minimal phagocytic activity (Figs.3.28 and 3.33). Thus, no differences in the cellular contents of murine peritoneal cavities inoculated with abscess-inducing mixtures containing either B. fragilis or B. vulgatus were observed at this stage.

However, at the end of the first 4.5 hrs of infection, more viable intracellular B. fragilis and E. coli, but not B. vulgatus, were found if bran was part of the abscess-inducing mixtures (Fig.3.25). Bran was phagocytosed in vivo (Figs.3.27b and 3.32) and its intracellular presence could possibly impede the bactericidal mechanisms of the neutrophils. Fewer peritoneal leukocytes were lavaged from mice 24 hrs after inoculation with B. fragilis or B. vulgatus, E. coli plus bran when compared to the number of leukocytes obtained 4.5 hrs after inoculation with mixtures containing bran (Table 3.3). Leukocytes and bacteria clumped in the peritoneal cavity in the presence of bran (Fig.3.27a) which could result in adherence of the clumps of cells and bacteria to peritoneal surfaces within 24 hrs. These cells would probably not be dislodged by lavage alone. This effect

of bran may be similar to the clumping factor possessed by some strains of S. aureus (Kapral et al., 1980).

The inoculation of abscess-inducing mixtures containing either B. fragilis or B. vulgatus appeared to have an adverse effect on the peritoneal macrophages 24 hrs after the initiation of infection (Fig.3.24). The macrophages had phagocytosed bacteria and other material, as evidenced by the contents of their phagosomes, but the macrophages were degenerating or dead after 24 hrs (Figs.3.29, 3.31, 3.34 and 3.36). Shands et al. (1974) demonstrated that endotoxin given IP had a cytotoxic effect on murine peritoneal macrophages. The administration of endotoxin caused a temporary depletion in the number of mononuclear phagocytes, and after 24 hrs the macrophages had a vacuolated appearance. Hammerström and Unsgaard (1979) showed endotoxin to be toxic to human monocytes. In the present study, regardless of the abscess-inducing mixture, the numbers of viable intracellular E. coli did not vary greatly over the 24 hr period (Fig.3.25) and E. coli phagocytosed in vivo for 1 or 24 hrs could not be killed in vitro (Table 3.6). This indicates a constant source of endotoxin exists in the peritoneal cavities of these inoculated mice. In contrast, Dunn et al (1985a) were not able to demonstrate that B. fragilis or E. coli, either together or separately, had an adverse effect on the peritoneal host defences of the rat.

In contrast to E. coli, there was a greater decline in the numbers of viable intracellular B. fragilis and B. vulgatus 24 hrs following the inoculation of abscess-inducing mixtures (Fig.3.25). The decrease in the number of viable intracellular Bacteroides species resulted in an increase in the ratio of peritoneal leukocytes to intracellular bacteria over the 24 hr period. B. vulgatus remained more susceptible than B. fragilis to in vitro phagocytic killing at these high ratios of leukocytes to bacteria after 24 hrs of in vivo

phagocytosis (Table 3.6). Neutrophils from one week old IA abscesses induced by AIM also cannot kill *in vitro* the *B. fragilis* and *E. coli* they had phagocytosed *in vivo* (Hart *et al.*, 1986b). However, neutrophils from peritoneal exudates of IA abscess-bearing mice could kill bacteria *in vitro* more efficiently than abscess-derived neutrophils. These results (Hart *et al.*, 1986b; Table 3.6) indicate the intracellular killing mechanisms of leukocytes involved in the initiation and development of IA abscesses are adversely affected in the environment of the peritoneal cavity and the resistance of some bacteria to phagocytic killing worsens the situation.

Thus, similar changes in the cellular content of the murine peritoneal cavity occurred following the inoculation of abscess-inducing mixtures containing either *B. fragilis* or *B. vulgatus*. Both *B. fragilis* and *B. vulgatus* were ingested by the phagocytes but their different susceptibilities to phagocytic killing may influence the further development of IA abscesses in this murine model.

CHAPTER 4

THE INTERACTION OF ABSCESS-INDUCING BACTERIA WITH MURINE PERITONEAL LEUKOCYTES IN VITRO

4.1 INTRODUCTION

Activation of the alternative and classical complement pathways and the presence of immunoglobulins facilitates the opsonization and phagocytic killing of Bacteroides species in vitro. Members of the genus Bacteroides are opsonized in human NS (Bjornson & Bjornson, 1978; Tofte et al., 1980; Joiner et al., 1981) and murine NS (Ellis & Barrett, 1982; Finlay-Jones et al., unpublished). However, isolated alternative complement pathway components had a predominant role in the deposition of C3bi fragments on the surfaces of B. fragilis and B. thetaiotaomicron (Bjornson et al., 1987).

The ability of phagocytes to kill Bacteroides species in vitro is not reflected in vivo. B. fragilis, in association with E. coli, persisted for up to ten weeks within IA abscesses in mice (Nulsen et al., 1983; Fig. 3.2). Furthermore, neutrophils from murine IA abscesses induced by B. fragilis, E. coli and bran are unable to significantly kill in vitro, in the presence of NS, the bacteria they had phagocytosed in vivo (Hart et al., 1986b). In the present study a murine strain of B. vulgatus did not survive in murine IA abscesses to the same extent as a murine strain of B. fragilis or B. fragilis ATCC 23745 (Fig. 3.2 and Fig. 3.3). Furthermore, differences between the numbers of viable B. fragilis and B. vulgatus within leukocytes were observed 1 hr after the IP inoculation of mice with abscess-inducing mixtures containing these bacteria (Fig. 3.25). Electron microscopic examination of murine peritoneal neutrophils revealed more damaged intracellular B. vulgatus than B. fragilis (Table 3.5). B. vulgatus phagocytosed in vivo were more susceptible than B. fragilis to phagocytic killing upon subsequent incubation of the peritoneal leukocytes with NS in vitro (Table 3.6). These results indicate that significant intracellular killing does not automatically follow the

phagocytosis of bacteria by phagocytes *in vivo* and that bacteria differ in their susceptibility to phagocytic killing.

Some phagocytosed bacteria resist the microbicidal mechanisms of neutrophils and macrophages by inhibiting the respiratory burst or degranulation. Inhibition of degranulation by bacteria in macrophages (Armstrong & Hart, 1971; Oberti *et al.*, 1981; Horwitz, 1983; Frehel *et al.*, 1986; Frehel & Rastogi, 1987; Sibley *et al.*, 1987) and neutrophils (Riley & Robertson, 1984; Bertram *et al.*, 1986) has been documented for several classical intracellular bacterial pathogens. If degranulation does occur, some bacteria survive either by escaping from the phagosome or by resisting oxygen-dependent killing and the toxicity of granule components.

The main hypothesis tested in the experiments described in this chapter was that *B. fragilis* survives within neutrophils because it inhibits fusion of the primary granules with phagosomes. The phagocytosis and killing by peritoneal leukocytes of the murine strain of *B. fragilis*, in defined conditions *in vitro*, was examined. Intracellular killing of *B. fragilis* was assessed in the presence or absence of ongoing phagocytosis of the bacteria by leukocytes. Results of ultrastructural studies on the location of the bacteria within neutrophils, the extent of granule fusion with bacteria-containing phagosomes and the susceptibility of the bacteria to intracellular degradation are presented. The effects of factors such as serum opsonins, the abscess-potentiating agent, the bacteria to leukocyte ratio and the availability of oxygen on the ability of *B. fragilis* to survive within neutrophils are described. Results on the *in vitro* phagocytosis and killing of *B. fragilis* by leukocytes are compared with murine strains of *B. vulgatus* and *E. coli*. Mixed populations of peritoneal leukocytes (Section 2.11) were used in the experiments described in this chapter.

4.2 RESULTS

4.2.1 Phagocytic Killing of Stationary Phase Bacteria Compared to Logarithmic Phase Bacteria

The aerobic phagocytic killing of stationary phase and logarithmic phase B. fragilis was compared in the presence of different opsonins (Table 4.1). There were no statistically significant differences between the phagocytic killing of stationary phase and logarithmic phase bacteria in either 10% NS, 10% IS or 10% NS plus 10% IS after 60 and 120 mins of incubation.

4.2.2 Cytological Assessment of the Phagocytosis of B. fragilis, B. vulgatus and E. coli

Cytocentrifuge smears were used to quantitate the involvement of neutrophils and mononuclear phagocytes in the phagocytosis of B. fragilis, B. vulgatus and E. coli in the presence of either 10% NS or 10% NS and 10% IS (Table 4.2). Since no attempt was made to remove extracellular bacteria, those bacteria in close proximity to the leukocytes may be either intracellular or attached to the leukocyte surface so the bacteria were referred to as leukocyte-associated.

B. fragilis and B. vulgatus were more closely associated with the peritoneal leukocytes than was E. coli (Fig. 4.1). Leukocytes were almost obscured by leukocyte-associated B. fragilis. Only 37% of neutrophils had leukocyte-associated E. coli in aerobic conditions, 24% of neutrophils had leukocyte-associated E. coli in anaerobic conditions. There were approximately three E. coli per neutrophil in aerobic and anaerobic conditions. In contrast, the numbers of leukocyte-associated B. fragilis and B. vulgatus were considered too great to be counted accurately, and more than

Table 4.1: Phagocytic Killing of Stationary Phase Bacteria Compared to Logarithmic Phase Bacteria in Aerobic Conditions

Opsonins	ΔB^a Stationary Phase 60 mins	Stationary Phase 120 mins	Logarithmic Phase 60 mins	Logarithmic Phase 120 mins
10% NS	-0.06 ± 0.11	-0.10 ± 0.11	-0.02 ± 0.05	0.01 ± 0.07
10% IS	-0.25 ± 0.19	-0.54 ± 0.35	-0.09 ± 0.04	-0.48 ± 0.03
10% NS + 10% IS	-0.96 ± 0.27	-1.31 ± 0.56	-1.10 ± 0.09	-1.82 ± 0.16

Stock cultures of B. fragilis were thawed and used immediately (stationary phase) or grown for 4 hrs in pre-reduced Schaedler broth (logarithmic phase) and used. The prepared bacteria ($5x10^5$) were combined in the assay tubes with peritoneal leukocytes ($5x10^6$) in the various opsonins and incubated at 37°C under rotation. Samples were taken at 0, 60 and 120 mins and the change in the viable count of the bacteria (ΔB) was determined.

[a] represents the mean ± 1 SD from 2-9 experiments

Table 4.2: Leukocyte-Associated Bacteria (LAB) at 60 mins

Opsonins	Bacteria	Neutrophils with LAB (%)[a] Aerobic	Neutrophils with LAB (%)[a] Anaerobic	Mononuclear phagocytes with LAB (%)[b] Aerobic	Mononuclear phagocytes with LAB (%)[b] Anaerobic
10% NS	B. fragilis	92 ± 10	100 ± 0	31 ± 18	33 ± 18
	B. vulgatus	87	ND[c]	7	ND
	E. coli	37 ± 11	24 ± 16	2 ± 2	6 ± 8
10% NS +	B. fragilis	99 ± 1	100 ± 0	37 ± 13	32 ± 14
10% IS	B. vulgatus	93	ND	9	ND
	E. coli	54 ± 3	41 ± 11	3 ± 3	0.4 ± 0.6

Bacteria ($5x10^8$) were incubated with $5x10^6$ peritoneal leukocytes in serum at 37°C under rotation in aerobic and anaerobic conditions. Cytocentrifuge smears were made after 60 mins and 200-300 leukocytes were counted per slide.

a (No. of neutrophils with LAB) / (Total no. of neutrophils) x 100

b (No. of mononuclear phagocytes with LAB) / (Total no. of mononuclear phagocytes) x 100

c ND = Not done

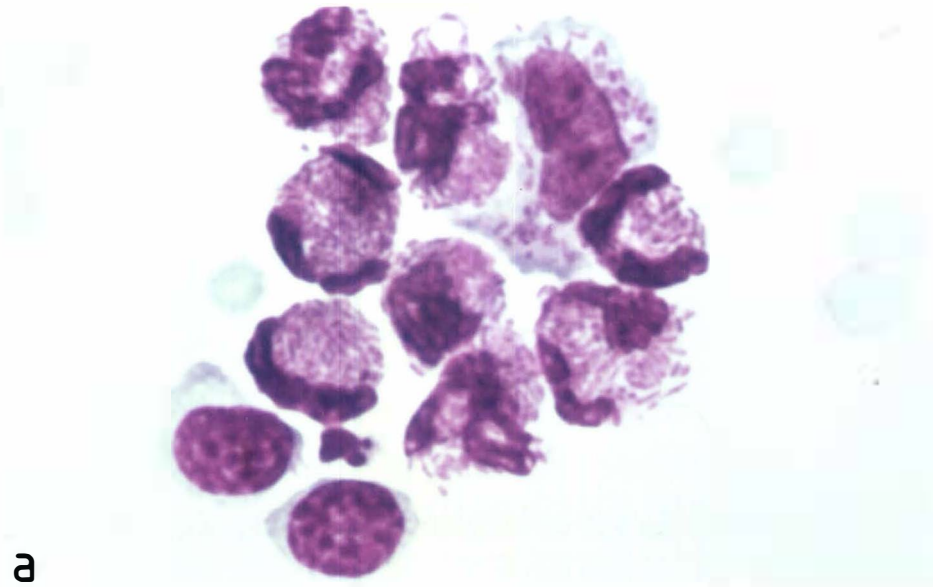
Figure 4.1: Phagocytosis of B. fragilis, B. vulgatus and E. coli

Bacteria ($5x10^8$) were incubated with $5x10^6$ peritoneal leukocytes in 10% NS and 10% of the appropriate IS at 37°C under rotation in aerobic conditions for 60 mins.

(a) Numerous B. fragilis appear to have been phagocytosed by neutrophils and one macrophage
x 1250.

(b) Apparently intracellular B. vulgatus are visible within neutrophils
x 1250.

(c) A few neutrophil-associated E. coli can be seen
x 1250.



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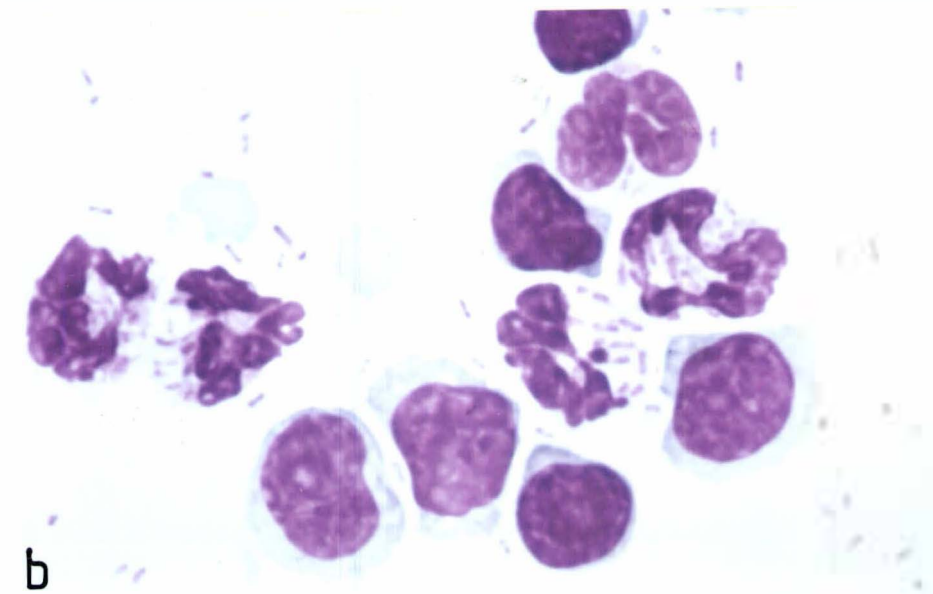

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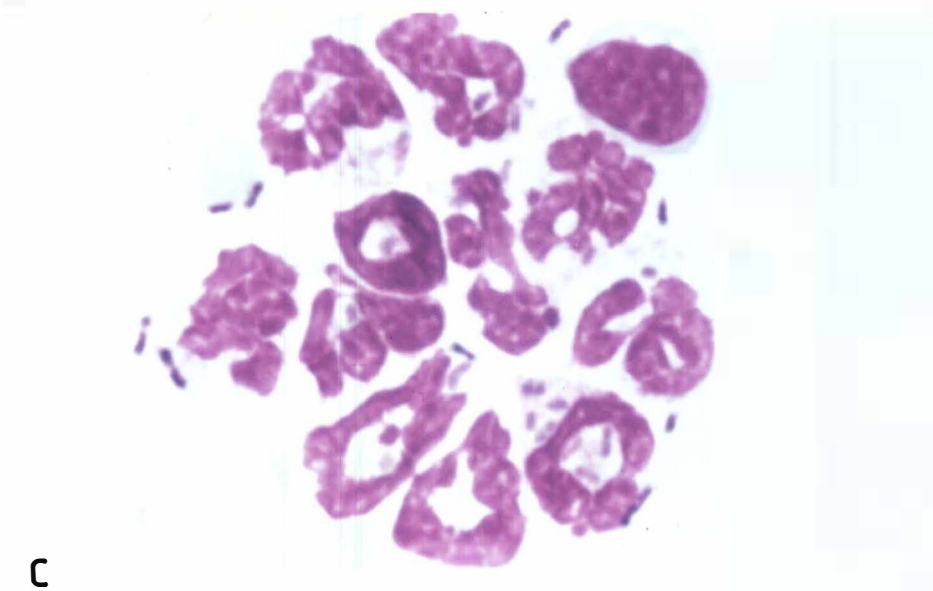


c

87% of neutrophils had leukocyte-associated bacteria in aerobic and anaerobic conditions. There was some involvement of mononuclear phagocytes in the phagocytosis of B. fragilis, B. vulgatus and E. coli in the rotating cell suspensions (Table 4.2).

In an experiment to test the effect of increased opsonins on phagocytosis, E. coli were pre-opsonized in 50% NS and 50% IS. The pre-opsonized bacteria were then incubated with peritoneal leukocytes in 1% NS to attain phagocytosis without intracellular killing, and the numbers of neutrophil-associated bacteria were monitored (Table 4.3). Although 71% of neutrophils had apparently phagocytosed E. coli, there were only two E. coli per neutrophil after 60 mins. However, upon heating E. coli at 80°C for 15 mins, there was more extensive phagocytosis of the organism by murine peritoneal leukocytes in 10% NS or 10% NS and 10% IS (Fig. 4.2).

4.2.3 Phagocytic Killing of B. fragilis, B. vulgatus and E. coli

The killing of B. fragilis, B. vulgatus and E. coli by murine peritoneal leukocytes, at a ratio of one bacterium per ten leukocytes, was determined aerobically in the presence of various opsonins (Fig. 4.3).

NS was not able to effect killing of B. fragilis by the peritoneal leukocytes after 120 mins (Fig. 4.3a). In 10% IS there was an 0.54 log decrease in the numbers of B. fragilis after 120 mins incubation with the peritoneal leukocytes. However, in the combined presence of NS and IS there was a 1.31 log decrease in the numbers of B. fragilis after 120 mins, indicating 95% of the bacteria had been killed. Phagocytic killing in NS and IS was significantly greater than in either NS or IS alone at both 60 and 120 mins ($p < 0.05$).

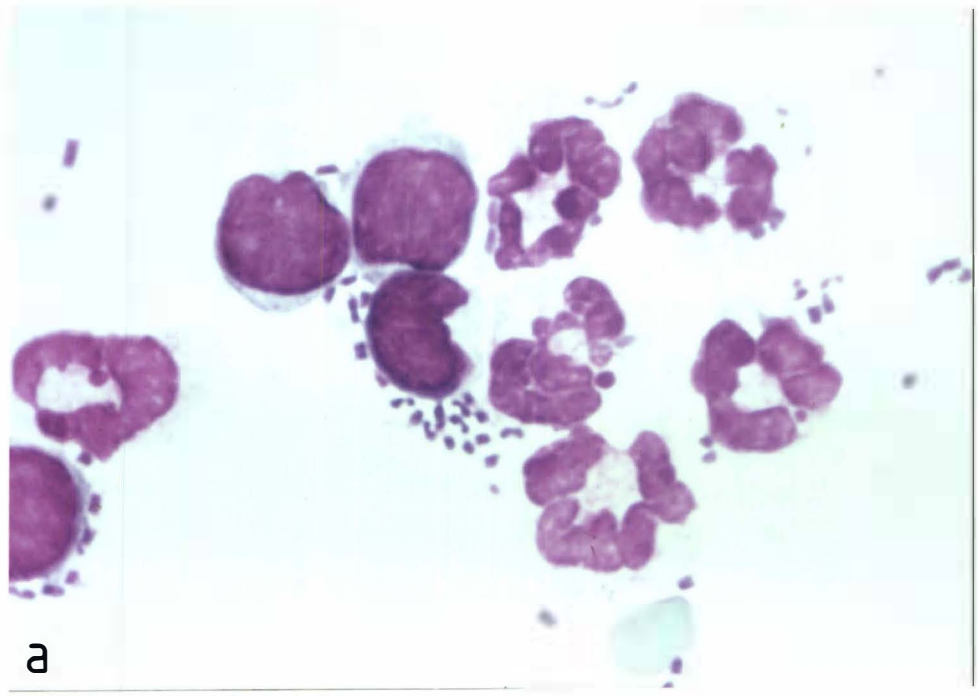
Table 4.3: Neutrophil-Associated *E. coli* After Pre-opsonization in 50% NS and 50% IS

Sampling Time (mins)	Neutrophils with *E. coli* (%)	No. *E. coli* per neutrophil
3	13.0	1.5
15	30.0	1.4
30	47.0	1.8
45	55.0	1.8
60	71.0	2.1

Pre-opsonized *E. coli* ($5x10^8$) were incubated with $5x10^6$ peritoneal leukocytes in 1% NS at 37°C under rotation in aerobic conditions. Cytocentrifuge smears were made over a 60 min period and 200 neutrophils were counted per slide

Figure 4.2: Phagocytosis of viable and non-viable *E. coli*

Viable (a) or heat-killed (b) *E. coli* were incubated with $5x10^6$ peritoneal leukocytes, at a ratio of 100 bacteria per leukocyte, in 10% NS and 10% IS at 37°C under rotation in aerobic conditions for 60 mins. More non-viable *E. coli* (arrowheads) than viable *E. coli* appear to have been phagocytosed by neutrophils.
(a) and (b) x 1250.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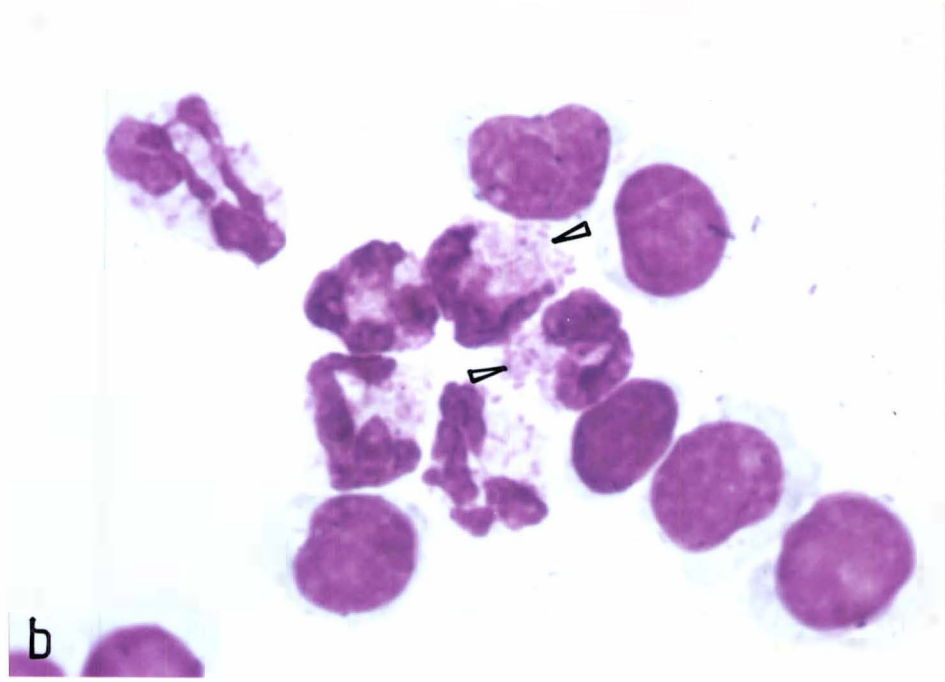
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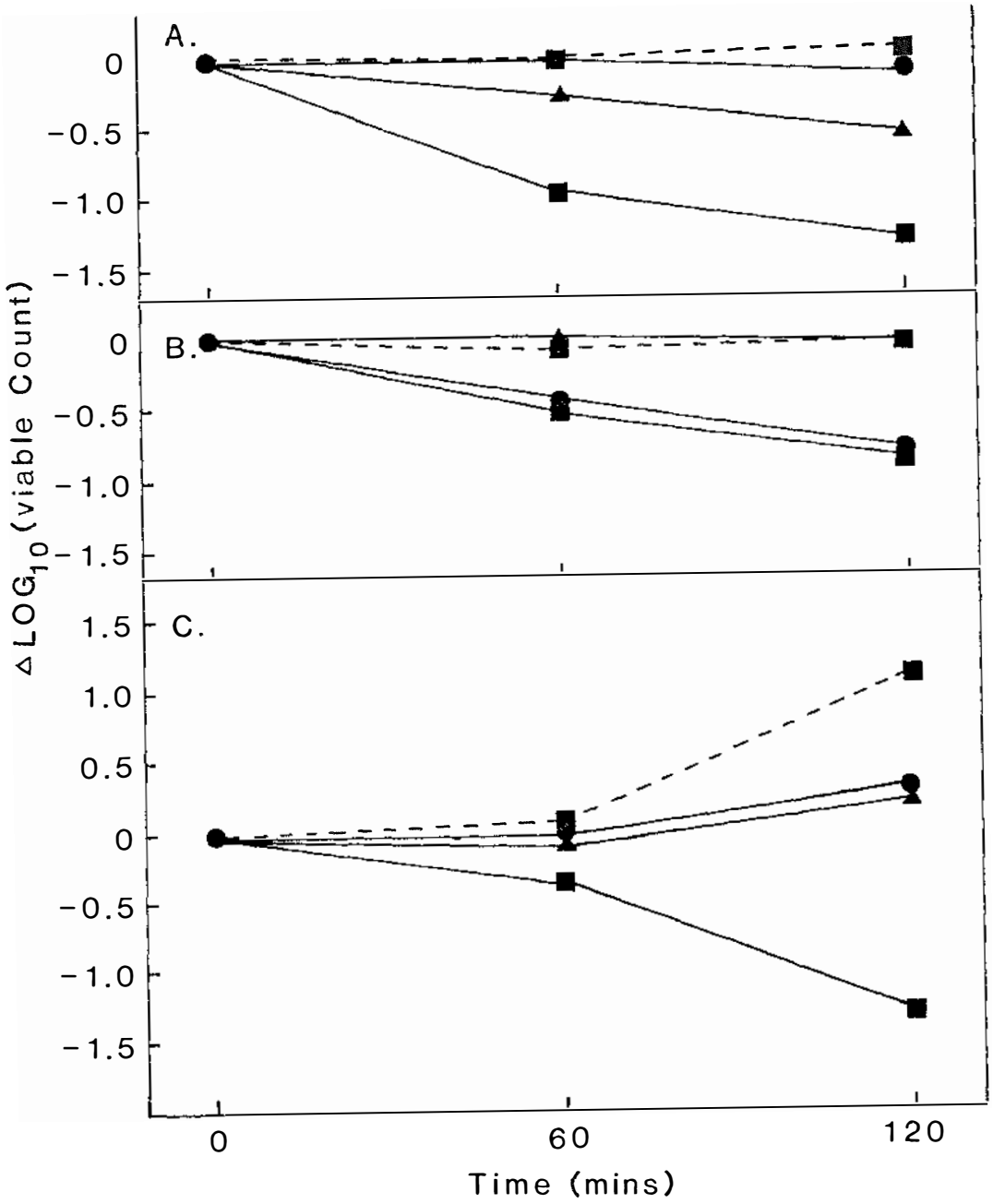

Figure 4.3: Phagocytic killing of bacteria in aerobic conditions. B. fragilis (A), B. vulgatus (B) and E. coli (C), at a concentration of $5x10^5$ cfu/ml, were incubated with (-) or without (---) $5x10^6$ peritoneal leukocytes in the presence of serum at 37°C under rotation. The serum was either 10% NS (●), 10% IS (▲) or 10% NS and 10% IS (■).

In the absence of peritoneal leukocytes B. fragilis was not killed in NS and IS.

NS was sufficient for the peritoneal leukocytes to kill 84% of B. vulgatus after 120 mins of incubation (Fig. 4.3b). In 10% NS more phagocytic killing of B. vulgatus than of B. fragilis occurred at 60 and 120 mins ($p < 0.05$). B. vulgatus was not killed by peritoneal leukocytes in the presence of IS and this lack of opsonic ability was also seen when killing was determined in the combined presence of NS and IS. The amount of phagocytic killing in NS and IS was not significantly different from that in NS alone. B. vulgatus was resistant to the effects of NS and IS in the absence of peritoneal leukocytes.

E. coli was not killed by peritoneal leukocytes in the presence of NS or IS (Fig.4.3c). In both instances growth of the bacteria occurred after 60 mins of incubation. The combined presence of NS and IS was required to promote significant phagocytic killing of 93% of the E. coli by 120 mins ($p < 0.05$). E. coli was not killed by NS and IS in the absence of peritoneal leukocytes.

4.2.4 The Effect of Oxygen on the Phagocytic Killing of B. fragilis and E. coli

The phagocytic killing of B. fragilis and E. coli by peritoneal leukocytes in NS and IS was determined in the presence and absence of oxygen (Table 4.4). Maximum killing of B. fragilis and E. coli was achieved in an aerobic environment when compared to an anaerobic situation ($p < 0.05$). In the case of B. fragilis the reduction in viable count was not due to oxygen sensitivity of the organism. Peritoneal leukocytes did kill small numbers of B. fragilis

Table 4.4: The Effect of Oxygen on the Phagocytic Killing of *B. fragilis* and *E. coli*

	ΔB^a			
	Aerobic		Anaerobic	
	60 min	120 min	60 min	120 min
B. fragilis	-0.96 ± 0.27	-1.31 ± 0.56	-0.54 ± 0.15	-0.21 ± 0.17
E. coli	-0.36 ± 0.15	-1.29 ± 0.35	-0.18 ± 0.13	-0.20 ± 0.43

Bacteria ($5x10^5$) were incubated with $5x10^6$ peritoneal leukocytes in 10% NS and 10% IS at 37°C under rotation in aerobic and anaerobic conditions. Samples were taken at 0, 60 and 120 mins and the change in the viable count of the bacteria (ΔB) was determined

[a] Represents the mean ± 1 SD from 4-9 experiments. Phagocytic killing of *B. fragilis* and *E. coli* was significantly greater in aerobic conditions when compared to anaerobic conditions ($p < 0.05$)

and E. coli anaerobically when compared with mixtures of bacteria and sera without peritoneal leukocytes ($p < 0.05$). However, after 60 mins in an anaerobic environment, B. fragilis can survive in opsonizing conditions which were sufficient for its phagocytic killing in aerobic conditions.

4.2.5 The Effect of Bacterial Concentration on the Activity of Peritoneal Leukocytes

4.2.5a Phagocytic Killing of B. fragilis and E. coli in the Presence of On-going Phagocytosis

In order to determine whether the peritoneal leukocytes can function in the presence of a large quantity of bacteria, as found in the infected peritoneal cavity, phagocytic killing assays were carried out in vitro at a ratio of 100 bacteria per leukocyte. The phagocytic killing of B. fragilis (Fig.4.4) and E. coli (Fig.4.5) was assayed in aerobic and anaerobic conditions. The killing of $5x10^5$ B. fragilis or E. coli by peritoneal leukocytes in maximal opsonizing conditions, i.e. 10% NS and 10% IS, was included for comparison.

In 10% NS and 10% IS there was significantly greater phagocytic killing of $5x10^5$ than of $5x10^8$ B. fragilis aerobically at 60 and 120 mins, and anaerobically at only 60 mins when results were expressed as the change in the viability ($\log_{10}$ cfu/ml) of the bacteria ($p < 0.05$). Both IS and NS were required for maximum phagocytic killing of $5x10^5$ B. fragilis (Fig.4.3a) so the phagocytic killing of $5x10^8$ B. fragilis was tested in 10% NS and 40% IS to supply an increased concentration of antibodies. In comparison with 10% NS alone, NS plus either 10% or 40% IS did cause a slight increase in the phagocytic

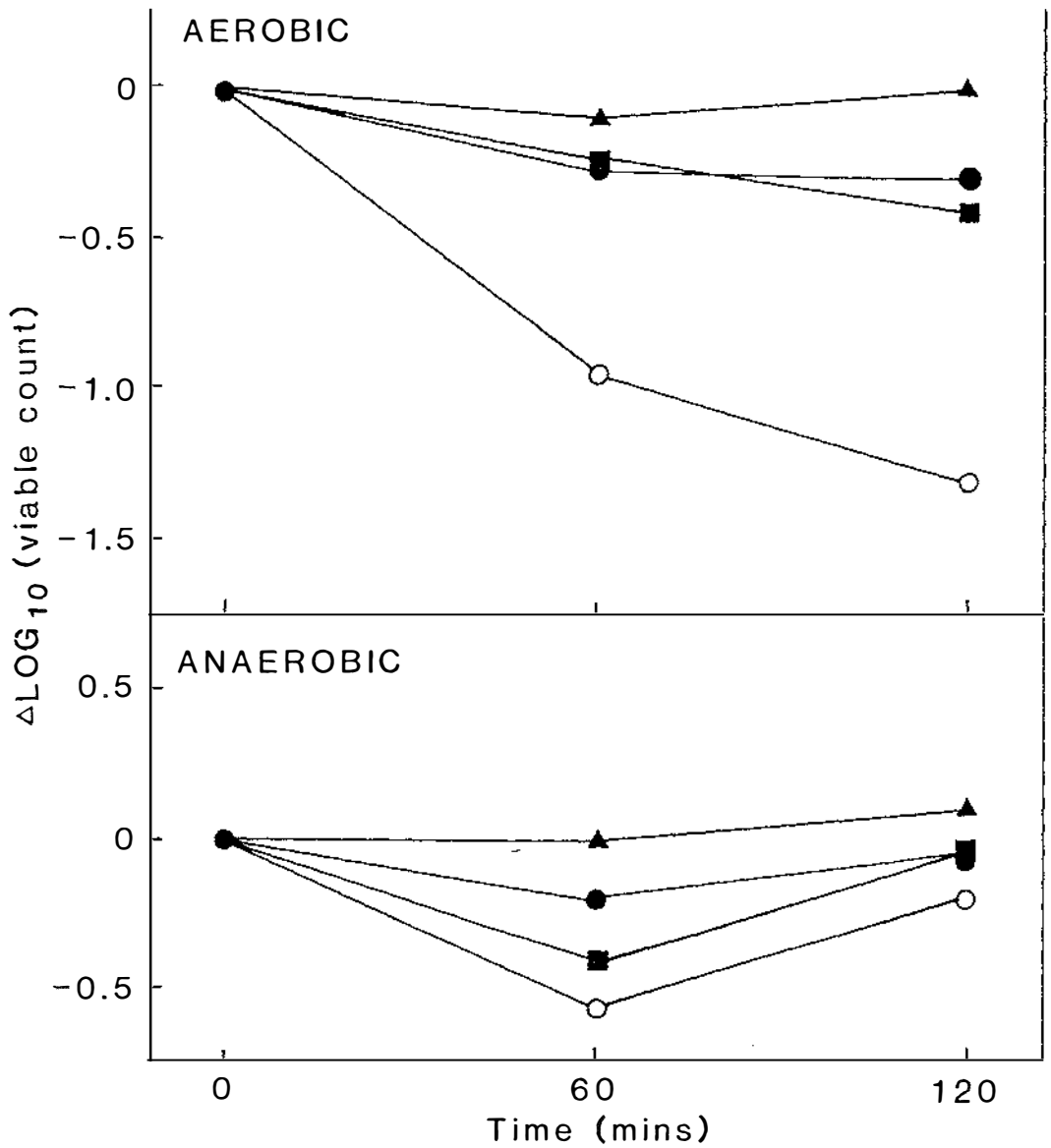

Figure 4.4: Effect of B. fragilis concentration on phagocytic killing in aerobic and anaerobic conditions. Peritoneal leukocytes ($5x10^6$) were incubated with $5x10^5$ B. fragilis (open symbol) or $5x10^8$ B. fragilis (closed symbols) in either 10% NS (▲), 10% NS and 10% IS (●, ○) or 10% NS and 40% IS (■) at 37°C under rotation

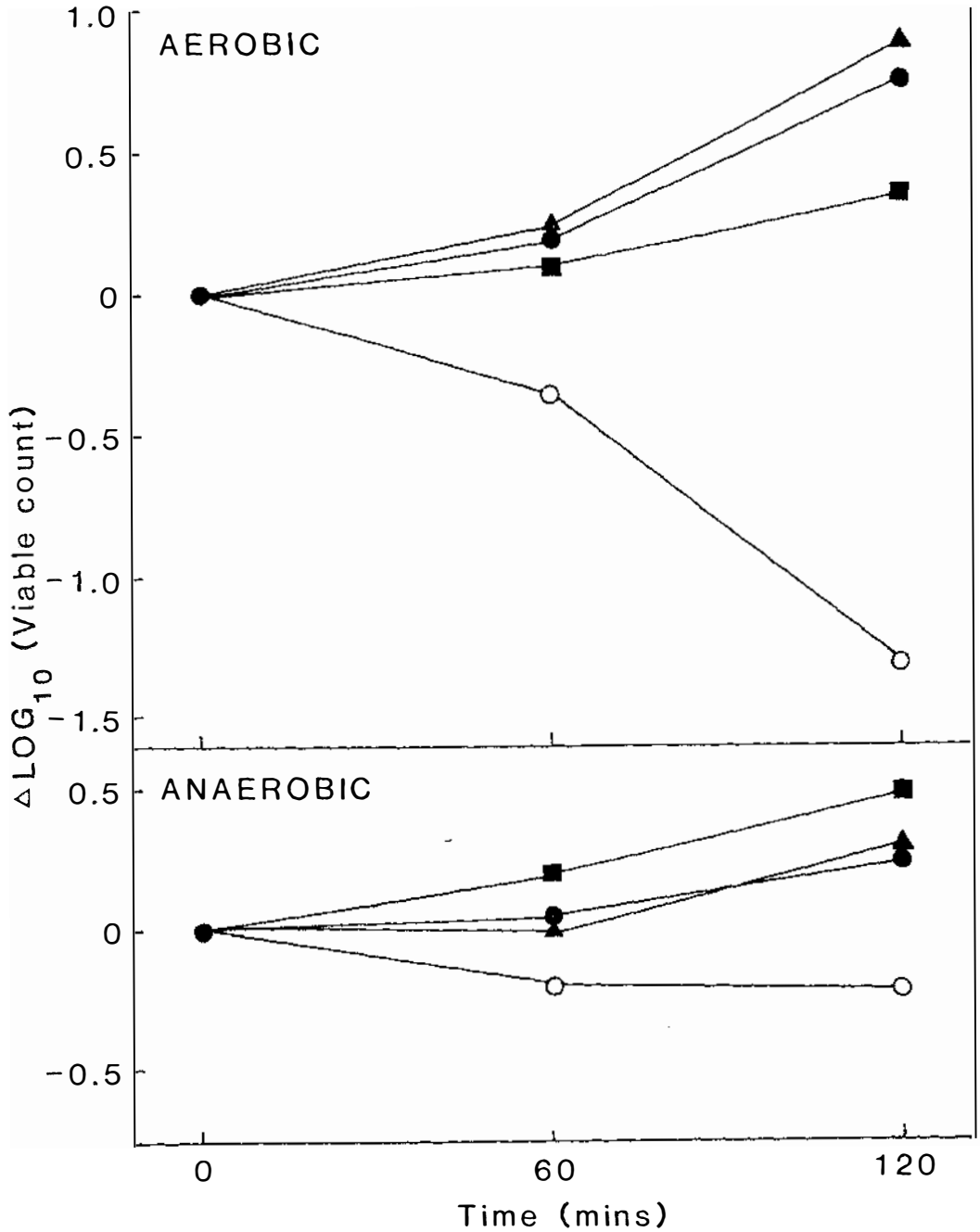

Figure 4.5: Effect of E. coli concentration on phagocytic killing in aerobic and anaerobic conditions. Peritoneal leukocytes ($5x10^6$) were incubated with $5x10^5$ E. coli (open symbol) or $5x10^8$ E. coli (closed symbols) in either 10% NS (▲), 10% NS and 10% IS (●, ○), or 10% NS and 40% IS (■) at 37°C under rotation

killing of B. fragilis after 120 mins in aerobic ($p < 0.05$) but not in anaerobic conditions. Phagocytic killing in 40% IS was not significantly greater than in 10% IS aerobically or anaerobically. Thus significant phagocytic killing at a high ratio of B. fragilis to peritoneal leukocytes was only observed aerobically and only if NS and IS were present.

In comparison to phagocytic killing at a ratio of one bacterium per ten peritoneal leukocytes, more phagocytic killing occurred at a ratio of 100 B. fragilis per leukocyte in terms of the total number of bacteria killed and the number of bacteria killed per leukocyte. However, in terms of the percentage of the total bacteria killed, a smaller proportion of B. fragilis was killed at the high ratio of bacteria to leukocytes than at the low ratio of B. fragilis to leukocytes (Table 4.5). In 10% NS and 10% IS, after 120 mins in aerobic conditions, 51% of $5x10^8$ B. fragilis were killed compared to 95% of $5x10^5$ B. fragilis. In anaerobic conditions, at both concentrations of bacteria, replication of B. fragilis had occurred at 120 mins which reduced the percentage of the total bacteria killed. Thus, more B. fragilis survived phagocytic killing at a ratio of 100 bacteria per peritoneal leukocyte.

Fig.4.5 shows the phagocytic killing of E. coli in aerobic and anaerobic conditions. Aerobically and anaerobically, there was significantly greater phagocytic killing of $5x10^5$ E. coli than of $5x10^8$ E. coli, in 10% NS and 10% IS, at 60 and 120 mins ($p < 0.05$). Peritoneal leukocytes had no significant effect on reducing the numbers of E. coli when compared to mixtures of $5x10^8$ E. coli and sera without peritoneal leukocytes.

Table 4.5: The Effect of *B. fragilis* Concentration on the Activity of Peritoneal Leukocytes

B. fragilis (cfu/ml)	Opsonins	O_2	Sampling Time (mins)	Total No. of Bacteria Killed[a]	% Total Bacteria Killed[b]	No. Bacteria Killed/ Leukocyte[c]
$5x10^5$	10% NS +	+	60	$3.08x10^5$	88	0.06
	10% IS	+	120	$3.34x10^5$	95	0.07
		-	60	$2.47x10^5$	71	0.05
		-	120	$1.59x10^5$	46	0.03
$5x10^8$	10% NS +	+	60	$1.90x10^8$	45	38
	10% IS	+	120	$2.14x10^8$	51	43
		-	60	$1.08x10^8$	36	22
		-	120	$2.70x10^7$	9	5
$5x10^8$	10% NS +	+	60	$1.57x10^8$	42	31
	40% IS	+	120	$2.12x10^8$	57	42
		-	60	$2.07x10^8$	59	41
		-	120	$3.30x10^7$	9	7

Bacteria were incubated with $5x10^6$ peritoneal leukocytes and sera at 37°C under rotation in aerobic and anaerobic conditions. Samples were taken at 0, 60 and 120 mins and viable counts on the total numbers of bacteria were performed

a To - Tn where Tn = 60 or 120 mins
b [(To - Tn)/To] x 100
c (To - Tn) / $5x10^6$ peritoneal leukocytes

Results represent the means of three experiments.
At both concentrations of *B. fragilis* growth of the bacteria had occurred in anaerobic conditions at 120 mins

4.2.5b Ultrastructural Observations on the Phagocytic Killing of B. fragilis and B. vulgatus

Ultrastructural studies on the fate of bacteria after phagocytosis were made in aerobic and anaerobic conditions *in vitro*, but there were no conspicuous differences noted between the two incubating conditions. The predominant leukocyte involved in the phagocytosis of the bacteria was the neutrophil and hence only the neutrophil response will be reported in detail here.

Intracellular *B. fragilis* were observed after 20 mins of phagocytosis in NS alone or with IS. In general, intracellular bacteria were of a similar appearance to the extracellular bacteria shown in Fig. 4.6. Fig.4.7a is an example of two neutrophils in 10% NS containing only 1-2 bacteria per phagosome. Some degranulation has occurred, as indicated by the presence of reaction product for peroxidase in a few phagosomes, but peroxidase-positive and peroxidase-negative granules still exist in the neutrophils' cytoplasm. The neutrophils look healthy. In contrast, Fig.4.7b shows a neutrophil with many intracellular bacteria contained mainly in one large phagosome which contains traces of reaction product for peroxidase. The majority of bacteria appear intact. *B. fragilis* was also phagocytosed by macrophages and eosinophils (Fig.4.8), although not every peritoneal leukocyte had visible intracellular bacteria.

The situation was similar at 60 mins, and after 120 mins in 10% NS both intact and necrotic neutrophils were observed. Intact neutrophils generally contained several phagosomes of varying sizes (Fig.4.9a). The phagosomes contained one or more bacteria, the majority of which did not show signs of damage. Reaction product for

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Figure 4.6: Extracellular B. fragilis after 20 mins in 10% NS

The bacteria have intact cell walls and dense cytoplasm. The bacteria possess a capsule (arrowheads).
x 40886.

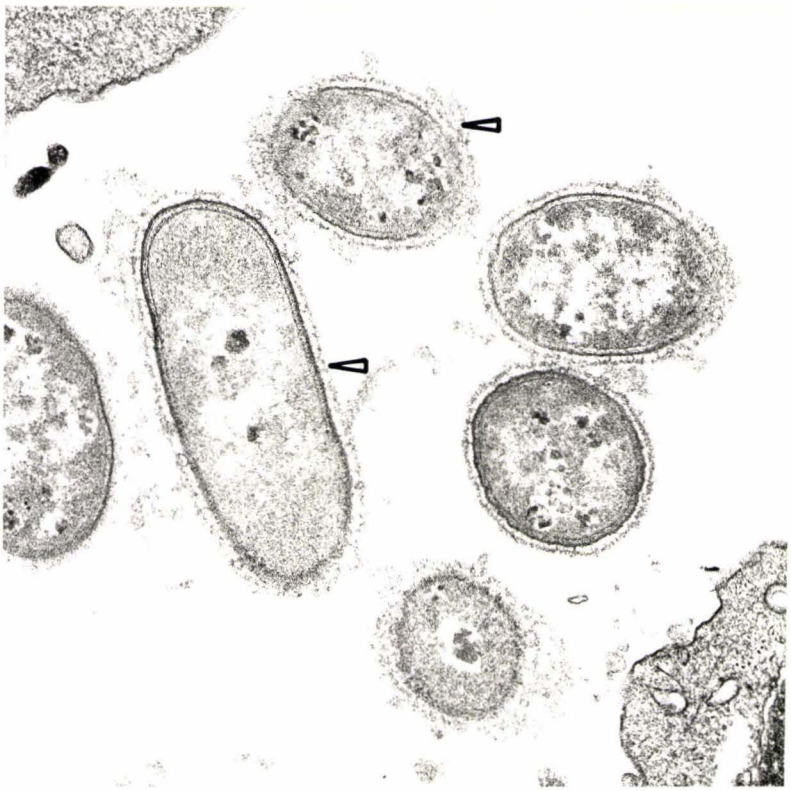

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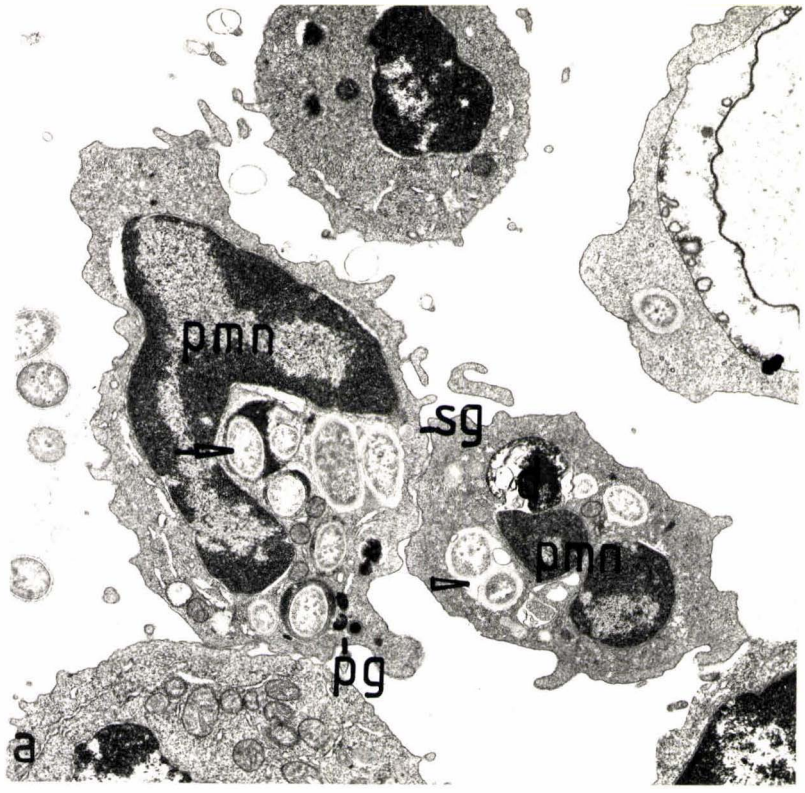
Figure 4.7: Phagocytosed *B. fragilis* after 20 mins in 10% NS

(a) Two neutrophils (pmn) containing 1-2 bacteria per phagosome. Bacteria are in peroxidase-positive (arrow) and peroxidase-negative (arrowhead) phagosomes. The bacteria appear intact. One phagosome contains debris (d). Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules are visible.
x 10029.

(b) A neutrophil with most bacteria contained in a large peroxidase-positive phagosome (p). The phagosome contains mostly intact bacteria (i) but some are slightly damaged (d).
nu = neutrophil nucleus.
x 12160.


pmn
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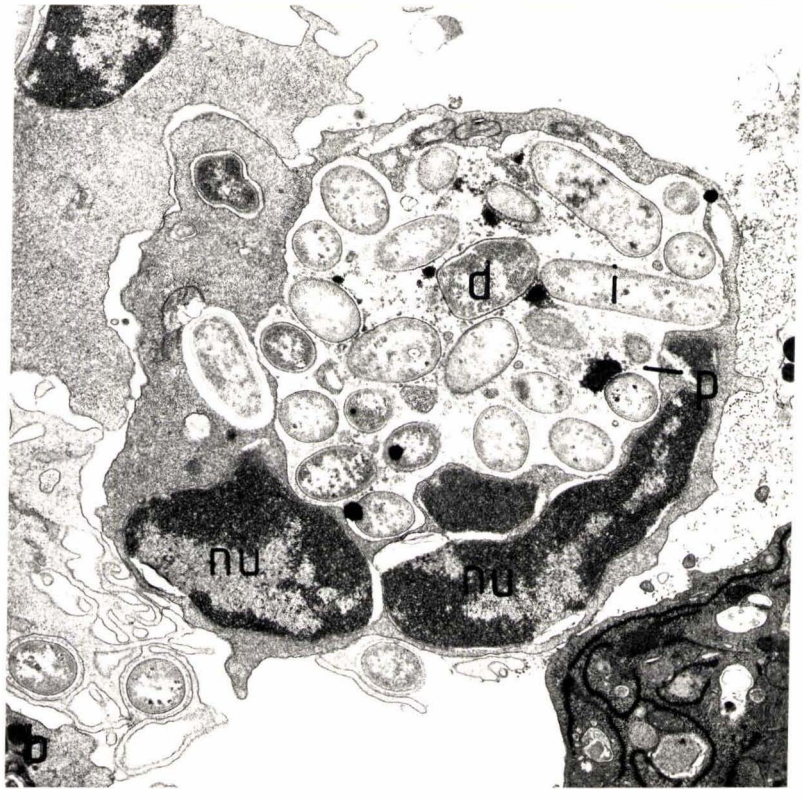
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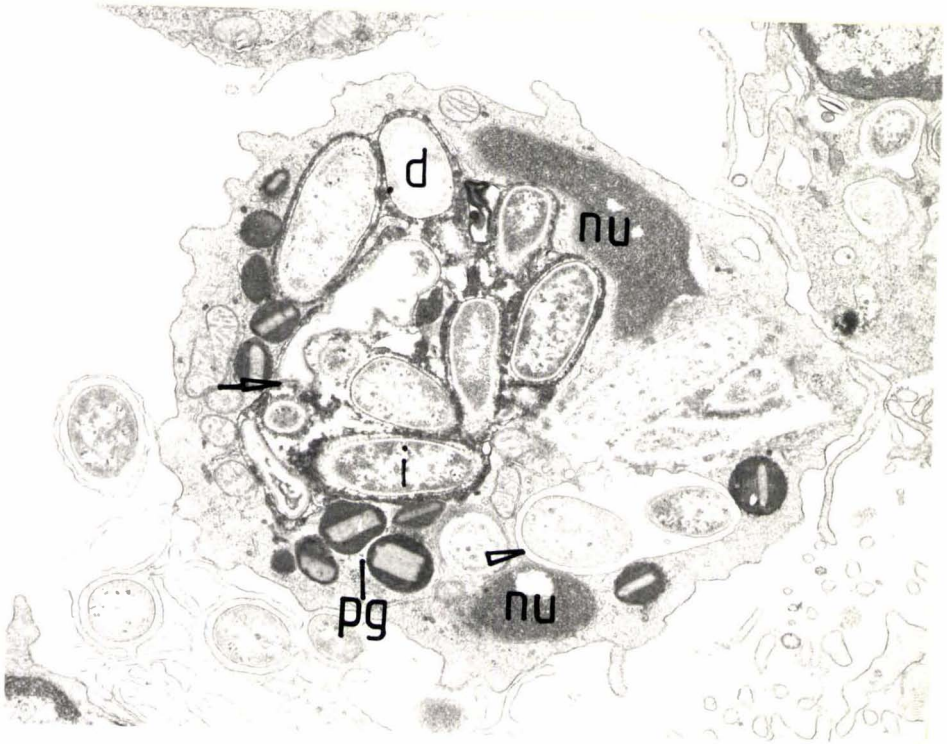
Figure 4.8: Phagocytosed B. fragilis after 20 mins in 10% NS

An eosinophil with peroxidase-positive granules (pg) in its cytoplasm. Intact (i) and slightly damaged (d) bacteria are contained within peroxidase-positive (arrow) and peroxidase-negative (arrowhead) phagosomes.
nu = eosinophil nucleus
x 14400.


d
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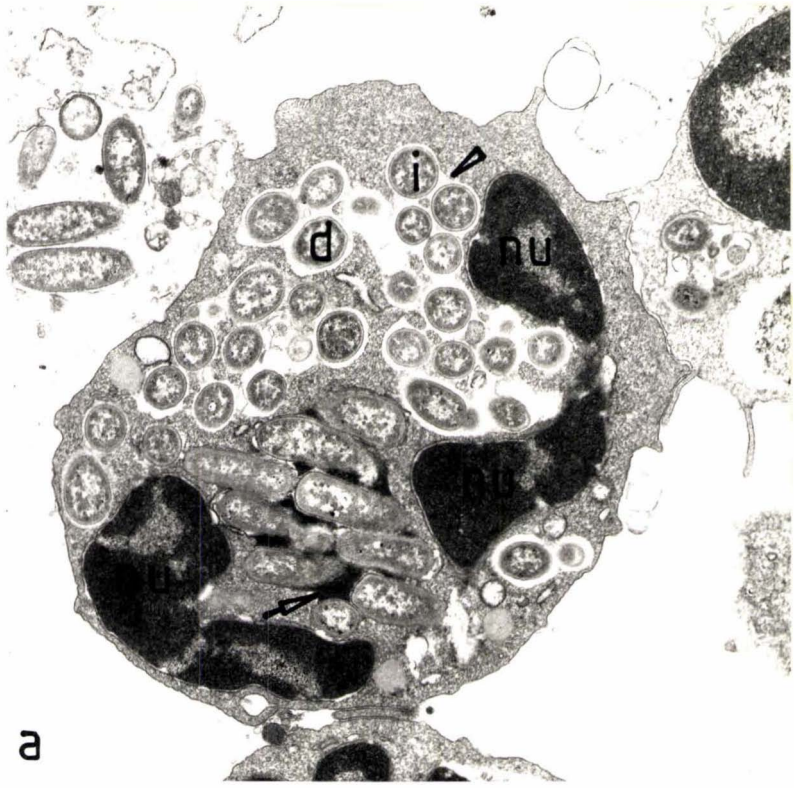
nu

Figure 4.9: Phagocytosed B. fragilis after 120 mins in 10% NS

(a) A neutrophil containing phagosomes of various sizes. Bacteria are in peroxidase-positive (arrow) and peroxidase-negative (arrow-head) phagosomes. Most bacteria are intact (i) but a few are slightly damaged (d). x 10697.

(b) An example of a necrotic neutrophil. Intact bacteria (i) are visible in peroxidase-positive (arrows) and peroxidase-negative (arrowhead) phagosomes. The membrane of one peroxidase-positive phagosome (p) appears to have disintegrated. x 14400.

nu = neutrophil nucleus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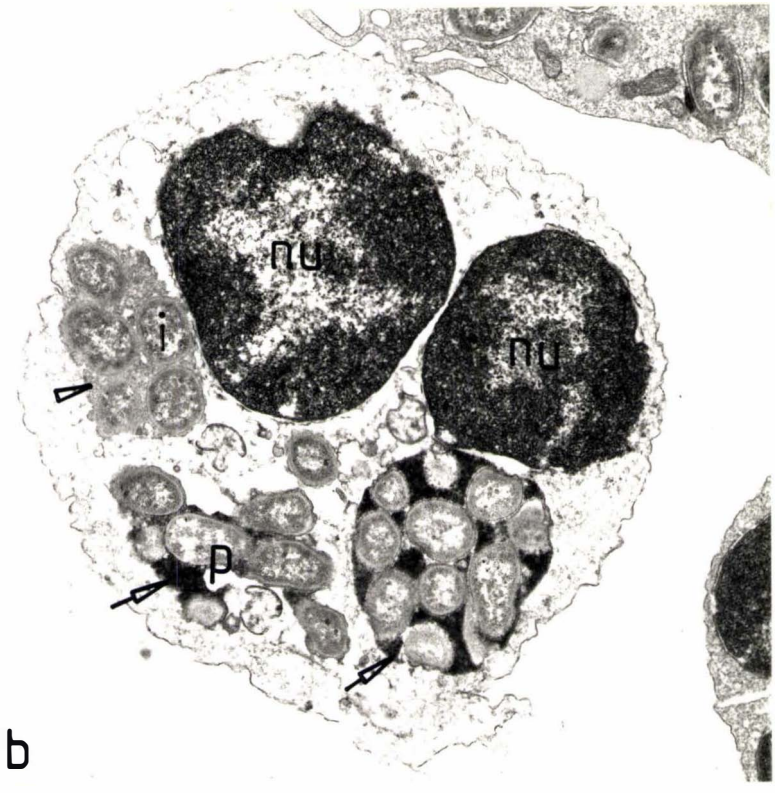
nu
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peroxidase was seen in some phagosomes. Entire bacteria were also observed in phagosomes of necrotic neutrophils, sometimes with reaction product for peroxidase (Fig.4.9b).

After phagocytosis in 10% NS and 10% IS, the appearance of the bacteria within neutrophils was like that in 10% NS at 20 mins. Fig.4.10a shows a neutrophil with phagosomes containing only a bacterium each, and most phagosomes show reaction product for peroxidase. Peroxidase-positive and peroxidase-negative granules are visible in the cytoplasm as well. A bacterium is damaged as shown by the vacuolated nature and decreased electron density of its cytoplasm. At the same time neutrophils with many bacteria in one large phagosome were seen (Fig.4.10b). Peroxidase-positive and peroxidase-negative granules can be seen in the neutrophil's cytoplasm. Although most bacteria look intact, a few may be damaged. The situation was unchanged at 60 mins.

Fig.4.11 shows neutrophils and macrophages involved in extensive phagocytosis after 120 mins in 10% NS and 10% IS. Neutrophils could be divided into three groups at 120 mins, based on the appearance of the phagosomes:

(a) Neutrophils with several small phagosomes containing 1-2 bacteria per phagosome (Fig.4.12a). Reaction product for peroxidase is visible in some phagosomes. Fig.4.12b illustrates the large phagocytic capacity of the neutrophils.

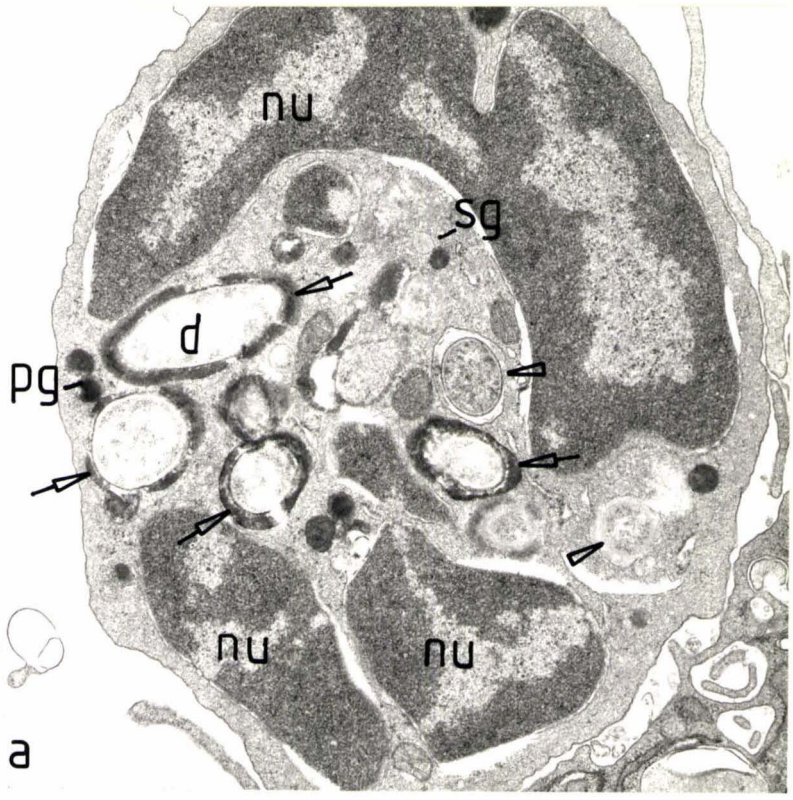
(b) Neutrophils with one to several larger phagosomes containing many intact bacteria plus evidence of some bacterial damage (Fig.4.13).

Figure 4.10: Phagocytosed B. fragilis after 20 mins in 10% NS and 10% IS

(a) A neutrophil with several peroxidase-positive phagosomes (arrows). Two peroxidase-negative phagosomes (arrowheads) are visible. A damaged bacterium (d) can be seen in one peroxidase-positive phagosome. Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules are in the cell's cytoplasm.
x 16611.

(b) A neutrophil with most bacteria contained within one large peroxidase-negative phagosome (arrowhead). Most bacteria are intact (i) but a few are damaged (d). Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules are visible in the cell's cytoplasm.
x 14400.

nu = neutrophil nucleus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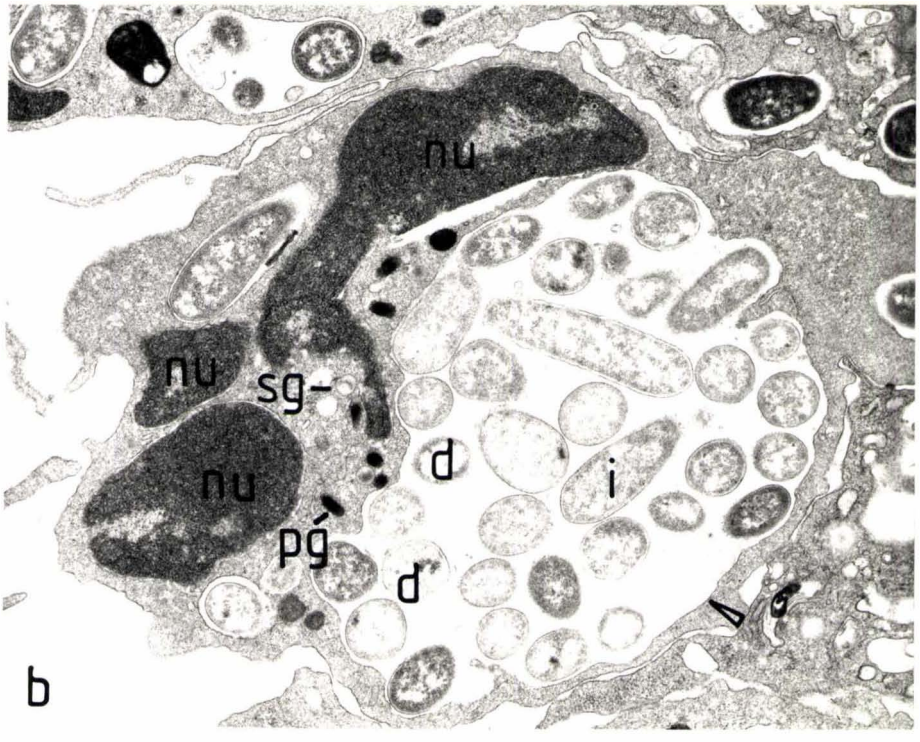
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Figure 4.11: Phagocytosed B. fragilis after 120 mins in 10% NS and 10% IS

A clump of neutrophils and one macrophage (ma) with many intracellular bacteria. The phagosomes (p) vary in size and contain one or more bacteria, of which most are intact.
x 4371.

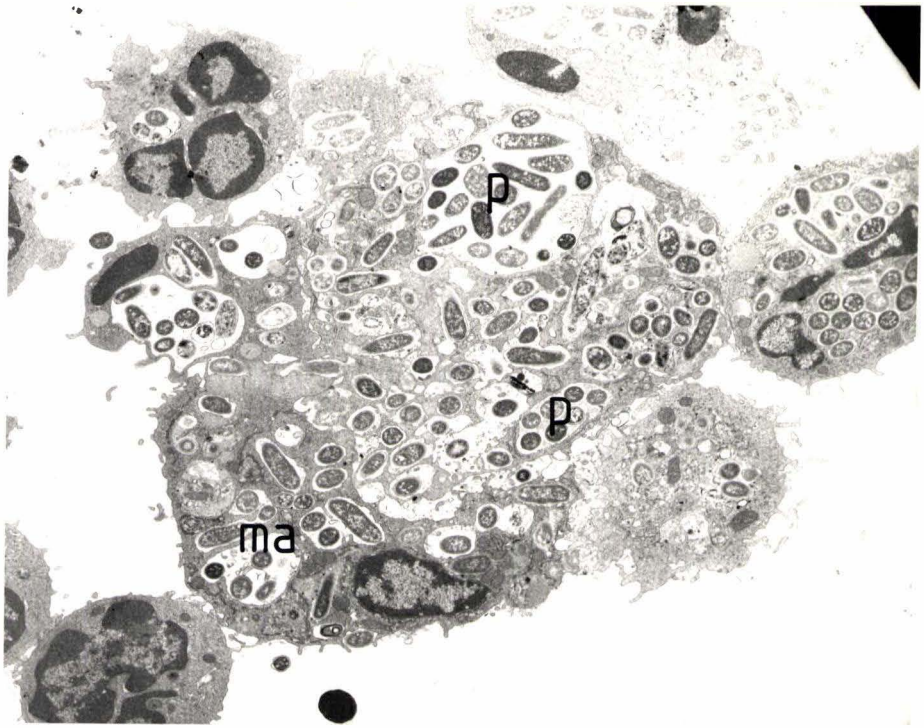
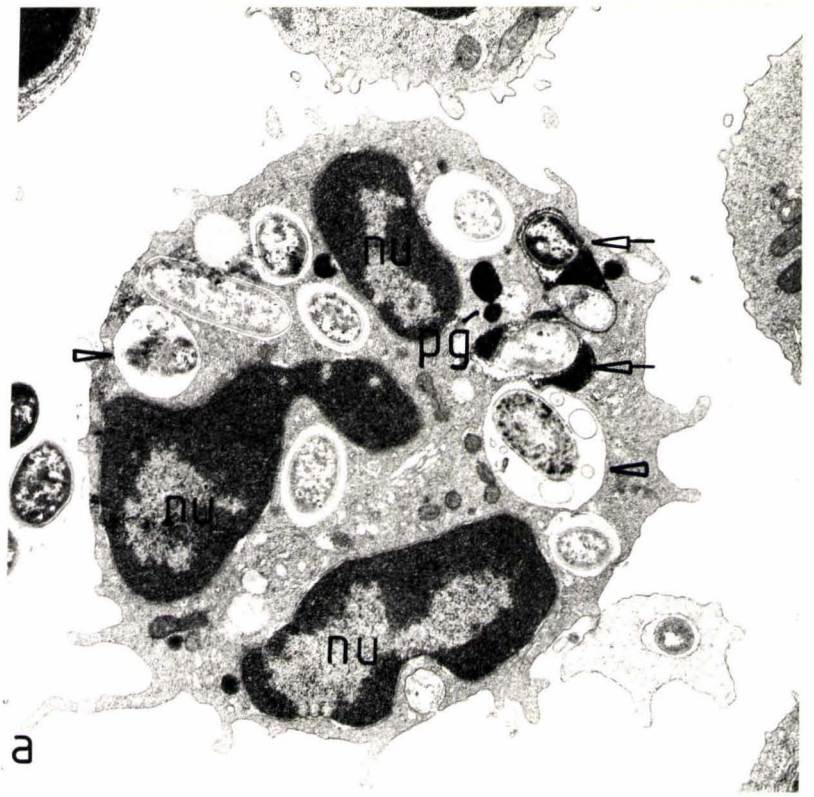

p
p
ma

Figure 4.12: Phagocytosed B. fragilis after 120 mins in 10% NS and 10% IS

(a) A neutrophil with several small peroxidase-positive (arrows) and peroxidase-negative (arrowheads) phagosomes, each containing 1-2 bacteria. Most bacteria are intact. A few peroxidase-positive primary granules (pg) are seen in the cell's cytoplasm.
x 12800.

(b) A neutrophil which has phagocytosed many bacteria. Each peroxidase-negative phagosome contains 1-2 intact bacteria.
x 8914.

nu = neutrophil nucleus



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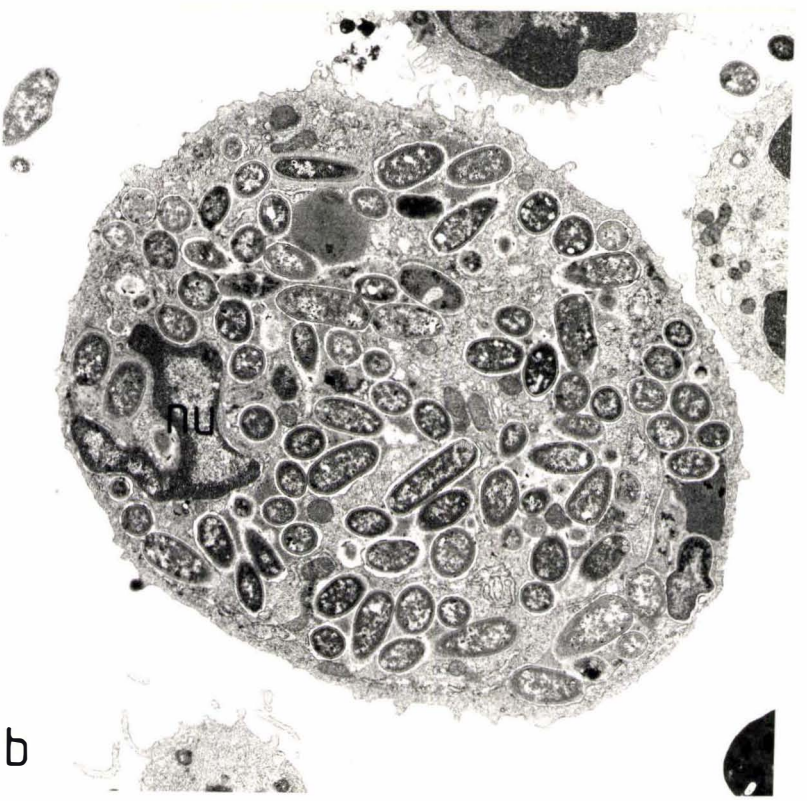


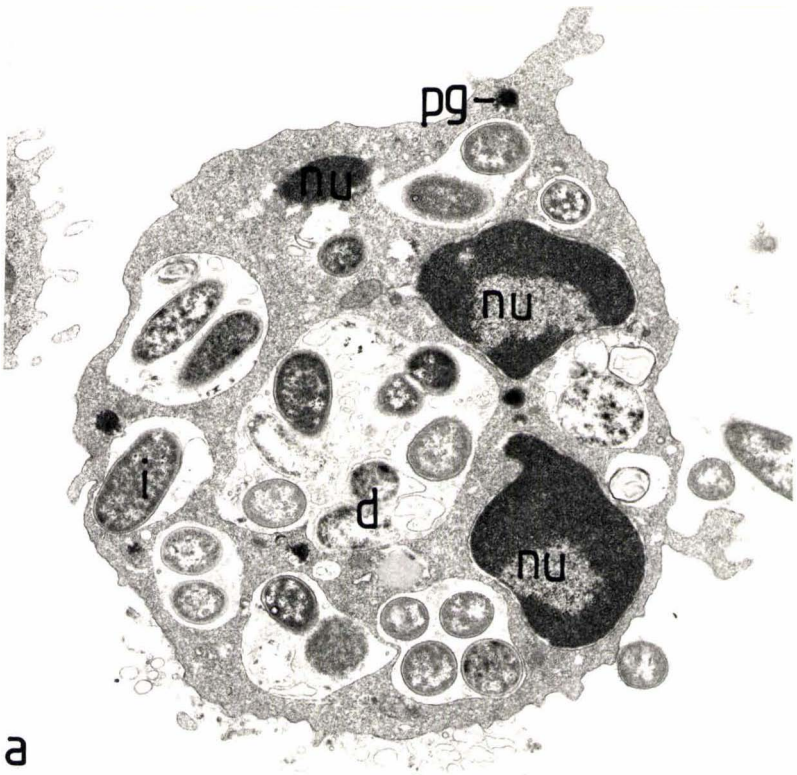
b

Figure 4.13: Phagocytosed B. fragilis after 120 mins in 10% NS and 10% IS

(a) A neutrophil with several phagosomes which are all peroxidase-negative. A few peroxidase-positive primary granules (pg) are in the cell's cytoplasm. Intact (i) and damaged (d) bacteria are seen within the phagosomes. x 13440.

(b) A neutrophil with one large phagosome and a few small phagosomes. All phagosomes are peroxidase-negative. Most bacteria appear intact (i), although bacterial debris (d) can be seen. x 12800.

nu = neutrophil nucleus



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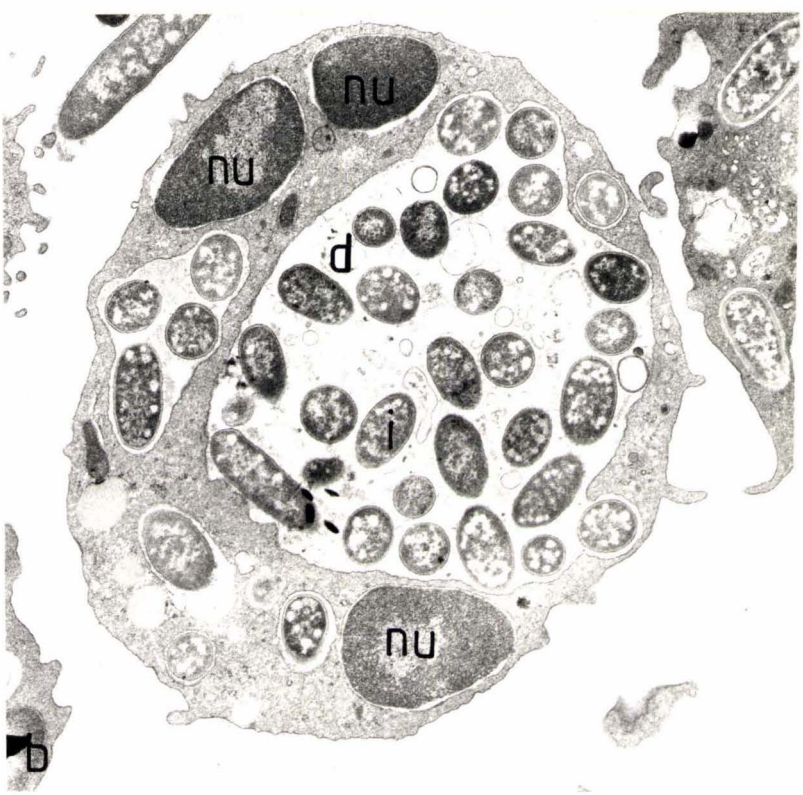


b

(c) Necrotic neutrophils, as indicated by the loss of integrity of the cytoplasmic membrane and decreased electron density of the cytoplasm, with large phagosomes. Intracellular bacteria can be seen and often the phagosomal membrane can be discerned (Fig.4.14a), although ultimately disruption of the neutrophil membranes can apparently lead to the release of intact, partially damaged and degraded bacteria into the extracellular milieu (Fig.4.14b).

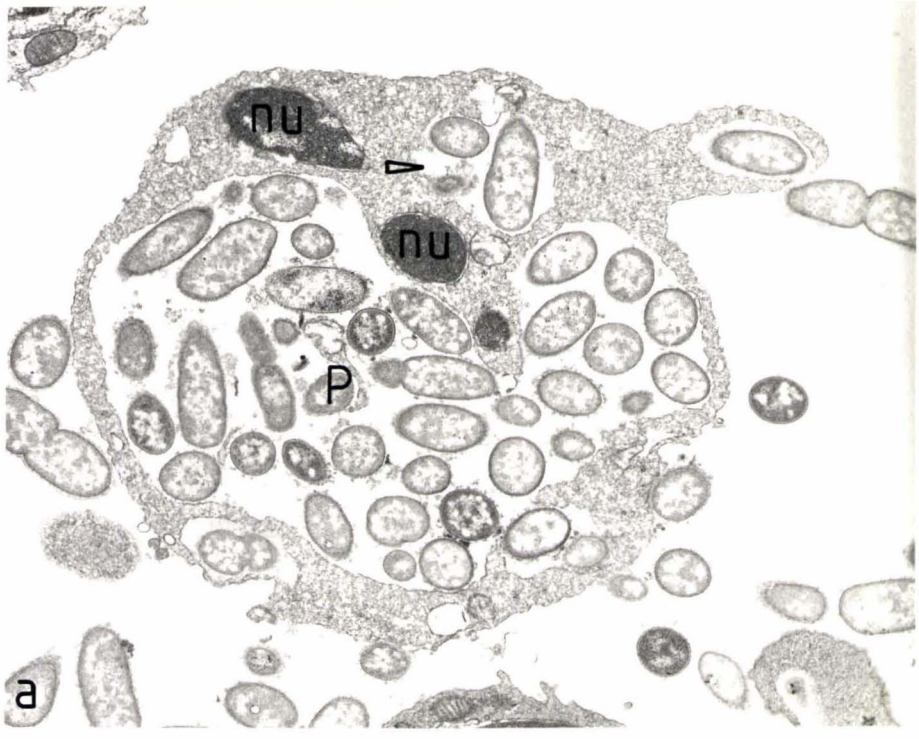
Fusion of neutrophil primary (peroxidase-positive) granules with phagosomes containing B. fragilis occurred, but the capacity of IS to enhance degranulation was not clear from visual observations. The effect of extracellular serum on the fusion of peroxidase-positive granules with phagosomes containing B. fragilis was assessed qualitatively, based on the disappearance of the cytoplasmic granules after the phagocytosis of the bacteria by peritoneal leukocytes for 30 mins in aerobic conditions (Table 4.6). When B. fragilis which were not pre-opsonized were phagocytosed in the presence of 1% NS, few intracellular bacteria were encountered in ultra-thin sections of neutrophils, and many peroxidase-positive and peroxidase-negative granules could still be seen in the cytoplasm (Fig.4.15a). In contrast, bacteria which had been pre-opsonized in 10% NS and 10% IS were frequently seen within neutrophils. Neutrophils, incubated with the pre-opsonized B. fragilis in either no serum or 10% HNS, showed reaction product for peroxidase in some phagosomes containing bacteria, but many peroxidase-positive and peroxidase-negative granules were visible in the neutrophils' cytoplasm (Fig.4.15b). When pre-opsonized B. fragilis were incubated in 10% NS, intracellular bacteria were seen in phagosomes of neutrophils and

Figure 4.14: Phagocytosed B. fragilis after 120 mins in 10% NS and 10% IS

(a) Necrotic neutrophil with most bacteria contained in one large phagosome (P). The membrane of one phagosome appears broken (arrowhead). Most intra-phagosomal bacteria are intact.
x 10029.

(b) A neutrophil whose cell membrane and phagosome membrane are disrupted (arrows). The bacteria originally enclosed in the large phagosome are mostly intact (i).
x 10029.

nu = neutrophil nucleus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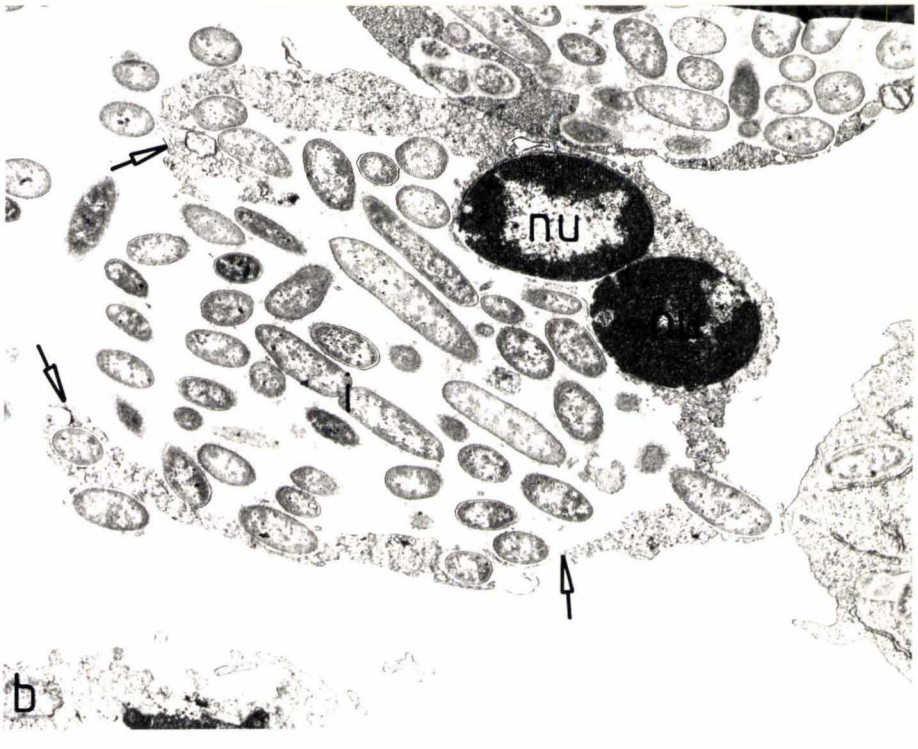
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Table 4.6: The Effect of Extracellular Serum on Degranulation

Pre-opsonization of *B. fragilis* in 10% NS and 10% IS	Extracellular Serum	Observations
-	1% NS	Few intracellular bacteria; many peroxidase-positive and -negative granules (Fig.4.15a)
+	-	Many intracellular bacteria; many peroxidase-positive and -negative granules (like Fig.4.15b)
+	10% HNS	Many intracellular bacteria; many peroxidase-positive and -negative granules (Fig.4.15b)
+	10% NS	Many intracellular bacteria; peroxidase-positive and -negative granules scarce (Fig.4.15c)

Peritoneal leukocytes ($5x10^6$) were incubated with $5x10^8$ non-opsonized *B. fragilis* in 1% NS or $5x10^8$ pre-opsonized *B. fragilis* in either no extracellular serum, 10% HNS or 10% NS for 30 mins in aerobic conditions at 37°C under rotation before the leukocytes were fixed, incubated in the peroxidase medium of Graham and Karnovsky (Materials and Methods) and processed for electron microscopy

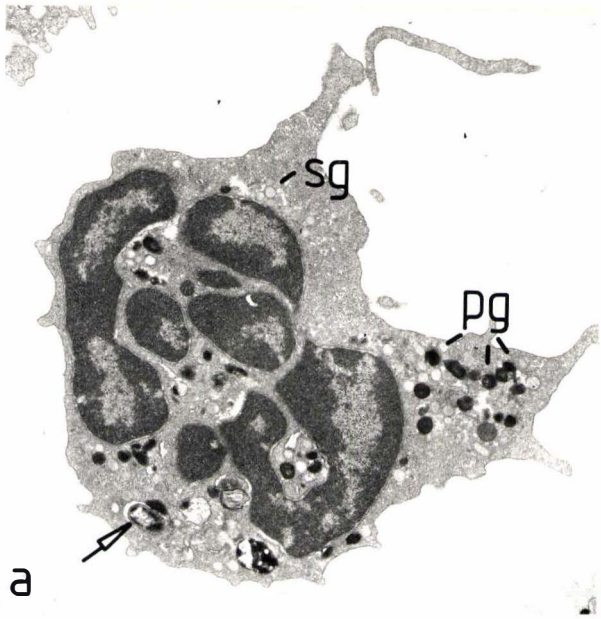
Figure 4.15: The effect of extracellular serum on degranulation after 30 mins of phagocytosis

(a) A neutrophil which was incubated with B. fragilis in 1% NS. The cell has many peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules in its cytoplasm. A bacterium can be seen in a peroxidase-positive phagosome (arrow). x 8914.

(b) A neutrophil which was incubated in 10% HNS with B. fragilis pre-opsonized in 10% NS and 10% IS. Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules are visible in the cell's cytoplasm. There are several intracellular bacteria (b) in peroxidase-negative (arrowheads) and peroxidase-positive (arrow) phagosomes. x 17486.

(c) A neutrophil which was incubated in 10% NS with B. fragilis pre-opsonized in 10% NS and 10% IS. One peroxidase-positive primary granule (pg) can be seen in the cell's cytoplasm. Mostly intact (i) and a few damaged (d) bacteria are visible in the cell's peroxidase-negative phagosomes. x 10400.

nu = neutrophil nucleus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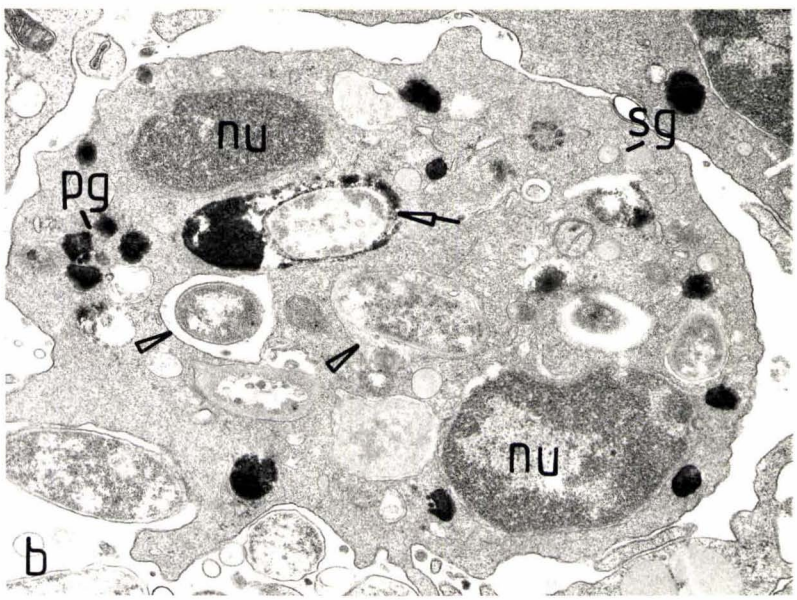
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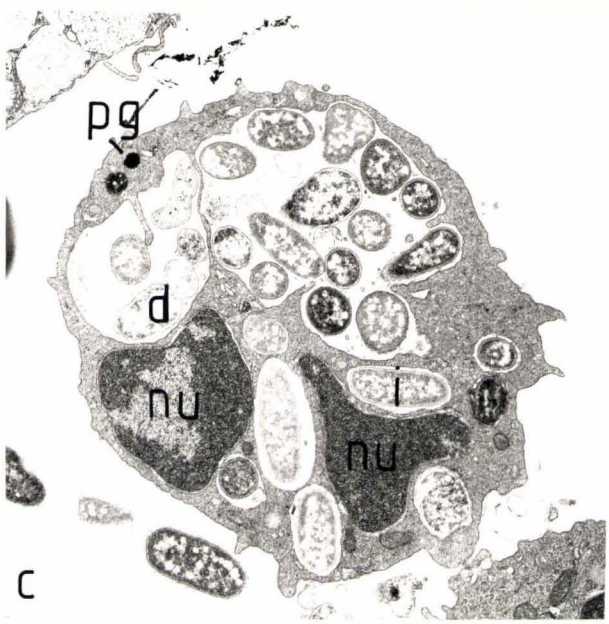
pg
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peroxidase-positive granules were scarce (Fig.4.15c).

Table 4.7 summarizes the results of an experiment to measure the degranulation of the primary granules in the presence of NS alone or with IS. Only 15% of the phagosomes containing B. fragilis examined after phagocytosis in 10% NS were peroxidase-positive. Of the 106 bacteria in the phagosomes only 12% were in peroxidase-positive phagosomes. After phagocytosis in 10% NS and 10% IS, 13% of the phagosomes containing B. fragilis were peroxidase-positive. Of the 392 bacteria in the phagosomes, only 10% were in peroxidase-positive phagosomes. The presence of NS and IS resulted in a larger number of phagosomes containing bacteria, but the presence of IS did not result in a large proportion of peroxidase-positive phagosomes. Thus antibody did not appear to promote fusion of primary granules with the phagosomes. The number of B. fragilis per phagosome, after phagocytosis in either 10% NS or 10% NS and 10% IS remained similar at 1.4 and 1.7 respectively.

When peritoneal leukocytes were exposed to $5x10^8$ B. vulgatus in 10% NS intracellular bacteria were scarce at 20 mins (Fig.4.16a). By 60 mins more intracellular B. vulgatus were encountered in the phagosomes of neutrophils. Fig.4.16b shows a neutrophil with damaged intraphagosomal bacteria. The bacteria show vacuolation and loss of electron density of their cytoplasm and disruption of their cell walls and membranes. Fragments of cell wall and membrane are visible inside the phagosomes.

After 20 mins in 10% NS and 10% IS B. vulgatus were found in the phagosomes of neutrophils, sometimes surrounded by reaction product for peroxidase (Fig.4.17a). The cytoplasm of the intracellular bacteria is vacuolated,

Table 4.7: Fusion of Neutrophil Primary Granules with Phagosomes Containing *B. fragilis*

Opsonins	No. Neutrophils Examined	No. Phagosomes Containing Bacteria	Peroxidase-Positive Phagosomes		No. Bacteria in Phagosomes	Bacteria in Peroxidase-Positive Phagosomes	
			No.	%		No.	%
10% NS	74	78	12	15	106	13	12
10% NS + 10% IS	48	230	29	13	392	40	10

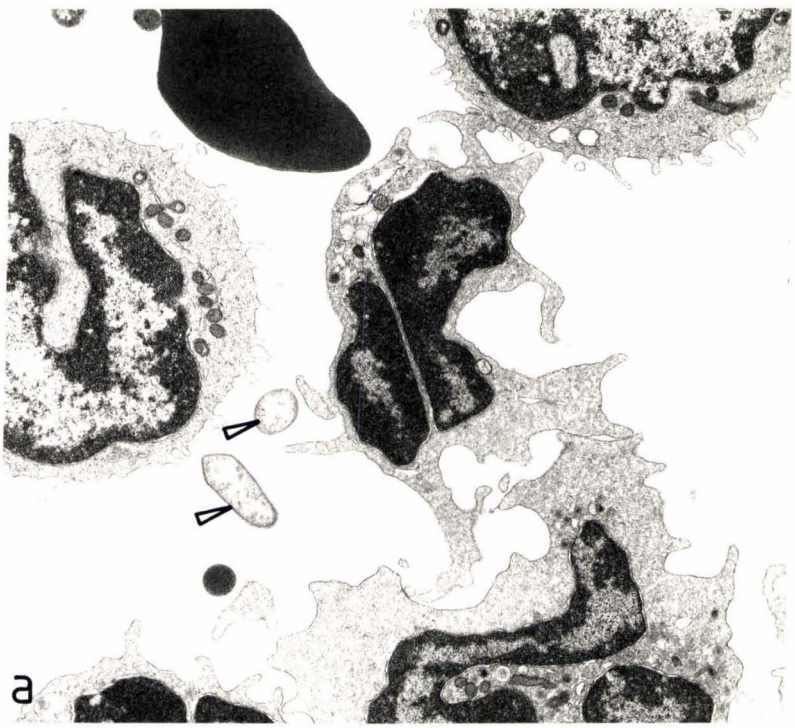
Peritoneal leukocytes ($5x10^6$) were incubated with $5x10^8$ *B. fragilis* in 10% NS and 10% NS plus 10% IS for 20 mins, in aerobic conditions at 37°C under rotation before the leukocytes were fixed, incubated in the peroxidase medium of Graham and Karnovsky (Materials and Methods) and processed for electron microscopy

Figure 4.16: Phagocytosed B. vulgatus in 10% NS

(a) After 20 mins of phagocytosis extracellular bacteria (arrowheads) are present and no intracellular bacteria are visible in this plane of section.
x 8023.

(b) After 60 mins of phagocytosis this neutrophil had phagocytosed several bacteria. Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules can be seen in the cell's cytoplasm. The bacteria are in peroxidase-negative phagosomes and all bacteria show signs of damage. Bacteria are vacuolated (v) and have damaged cell walls and membranes (arrow). The remains of a bacterium are visible in one phagosome (arrowhead).
x 14400.

nu = neutrophil nucleus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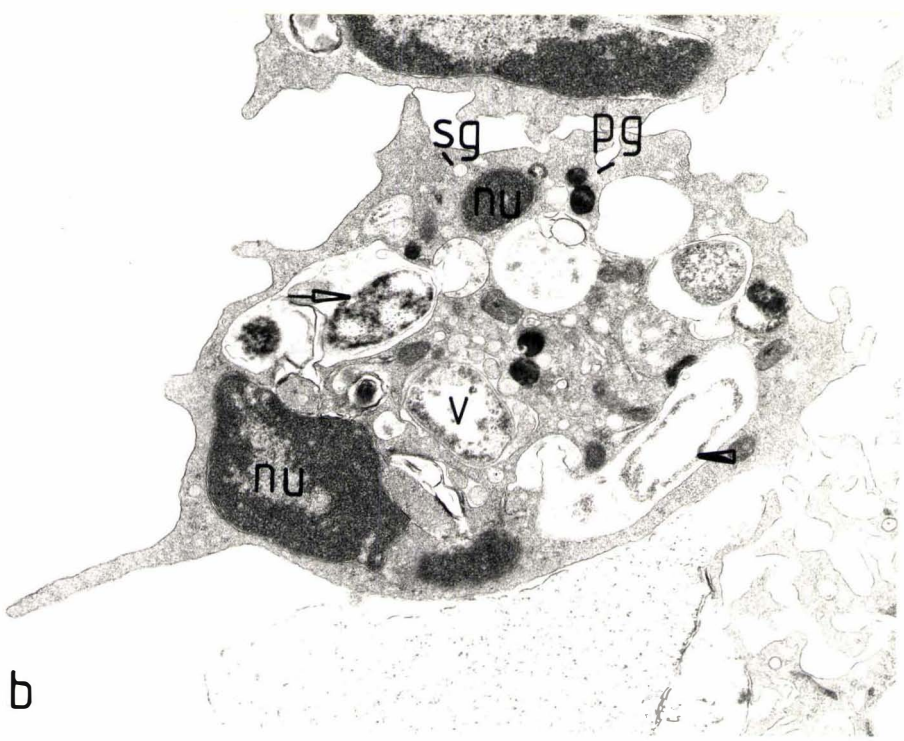
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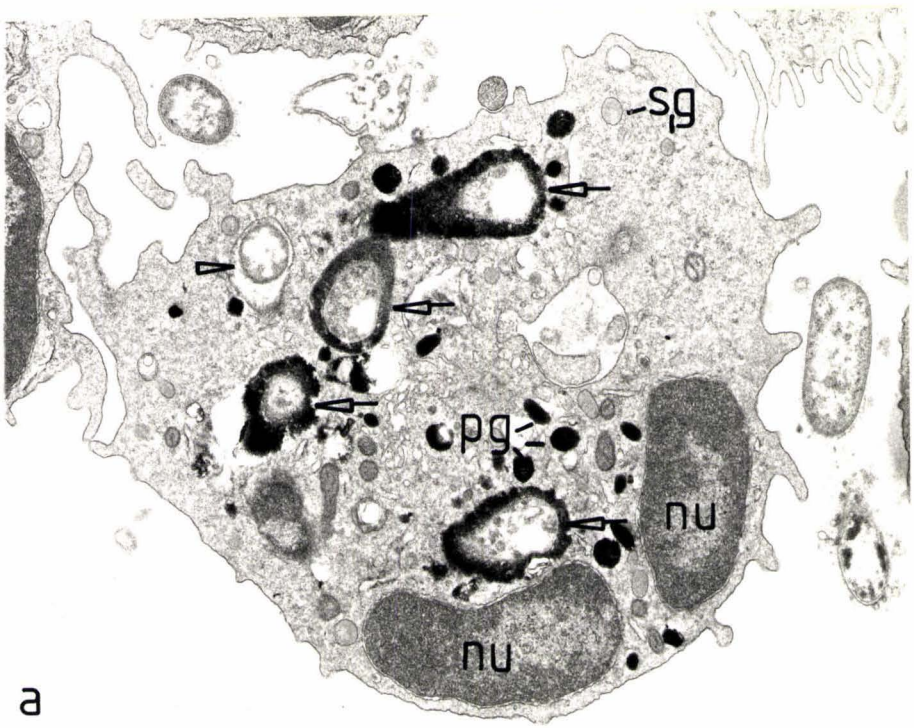
Figure 4.17: Phagocytosed B. vulgatus in 10% NS and 10% IS

(a) After 20 mins of phagocytosis this neutrophil contains bacteria in peroxidase-positive phagosomes (arrows). The bacteria are damaged, as indicated by their vacuolated cytoplasm. A bacterium can be seen in a peroxidase-negative phagosome (arrowhead). Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules can be seen in the cell's cytoplasm.
x 14400.

(b) After 60 mins of phagocytosis degraded bacteria (B) are visible in a phagosome of this neutrophil. Bacteria can be seen in other phagosomes. All phagosomes in this section are peroxidase-negative.
x 14400.

nu = neutrophil nucleus



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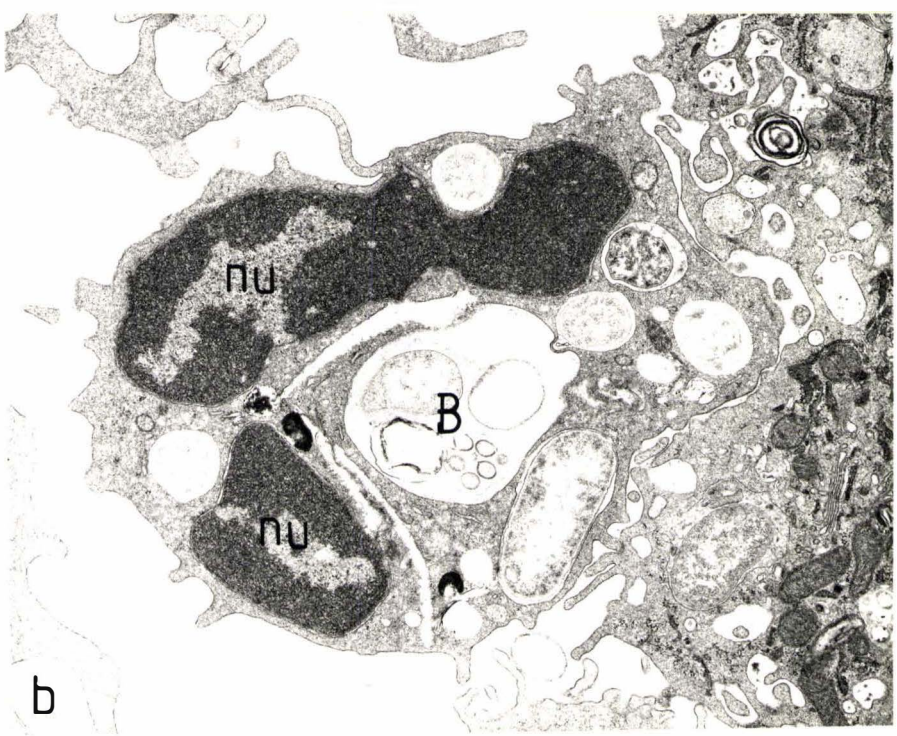

b

a sign of damage. Many peroxidase-positive and peroxidase-negative granules can still be seen in the neutrophil's cytoplasm. Evidence of intraphagosomal damage to the bacteria was still visible after 60 mins in 10% NS and 10% IS (Fig.4.17b). In general, more frequent evidence of the destruction of intracellular B. vulgatus than of intracellular B. fragilis was observed.

4.2.5c Ultrastructural Observations on the Phagocytic Killing of E. coli

The fate of intracellular E. coli phagocytosed in aerobic and anaerobic conditions was studied by electron microscopy. As noted for B. fragilis, there were no apparent differences observed between the two incubating conditions. E. coli was not as readily phagocytosed as B. fragilis (Table 4.2) and therefore it was not seen as frequently as B. fragilis in ultra-thin sections of neutrophils.

After phagocytosis in the presence of either 10% NS or 10% NS and 10% IS for 20 mins, intracellular E. coli were rarely encountered in neutrophils. In general, neutrophils with intact cytoplasmic peroxidase-positive and peroxidase-negative granules were observed, although the neutrophils were often vacuolated (Fig.4.18). The vacuoles often contained material of varying electron density. The situation was similar after 60 mins in 10% NS alone.

Intracellular E. coli were more frequently observed after 60 mins of phagocytosis in 10% NS and 10% IS (Fig.4.19a). Only 1-2 E. coli per phagosome were seen, and they were either intact (Fig.4.19a) or damaged (Fig.4.19b), as indicated by the vacuolation of the bacterial cytoplasm

Figure 4.18: Phagocytosed *E. coli* after 20 mins in 10% NS. The neutrophil has peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules in its cytoplasm. Vacuoles (v) containing debris can be seen. No intracellular bacteria can be seen in this plane of section.
x 13600.

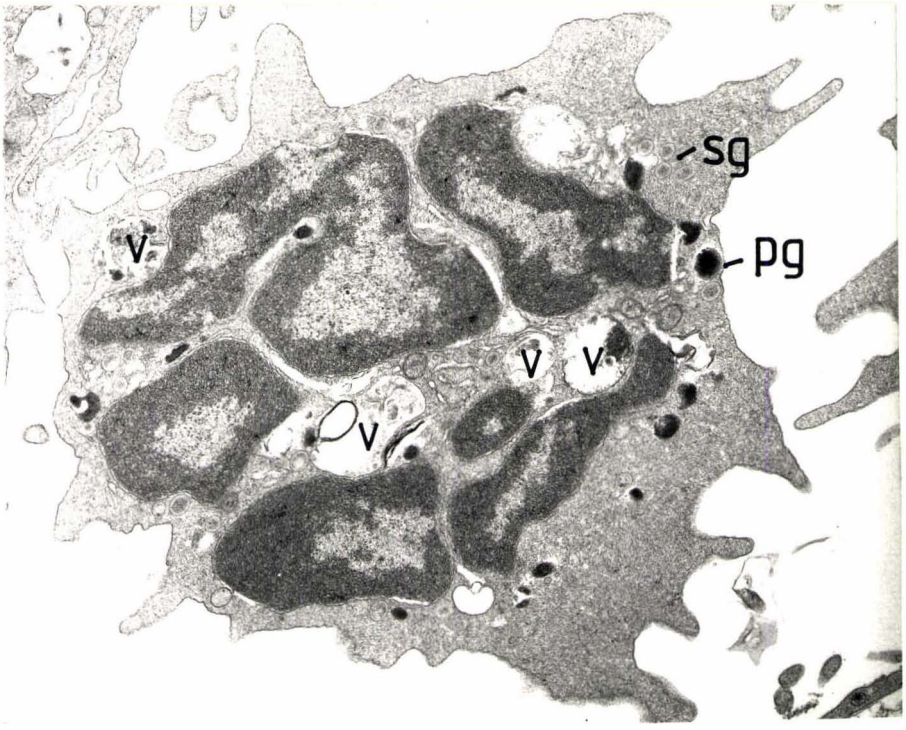
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Figure 4.19: Phagocytosed E. coli after 60 mins in 10% NS and 10% IS

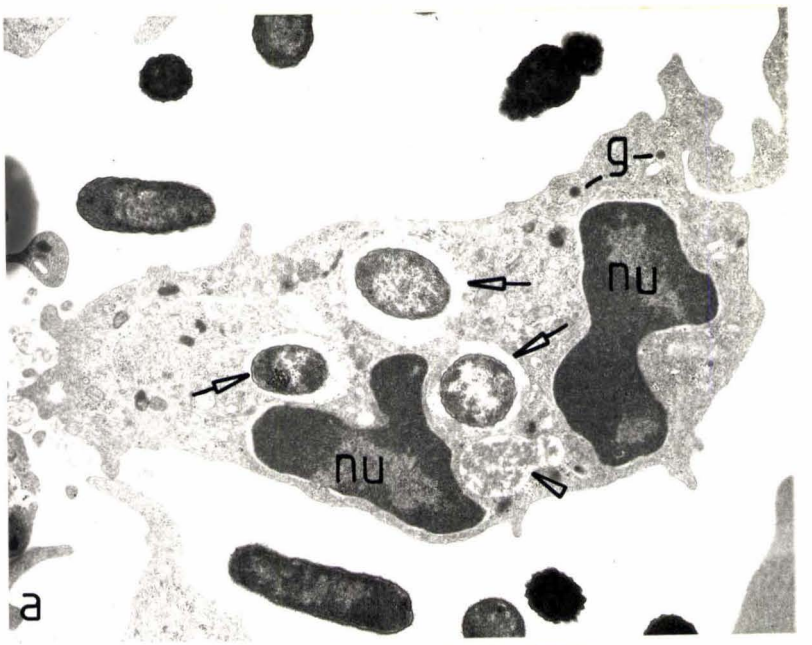
(a) A neutrophil containing intact bacteria in phagosomes (arrows). One phagosome contains debris (arrowhead). The cytoplasm of the cell contains granules (g).
x 12800.

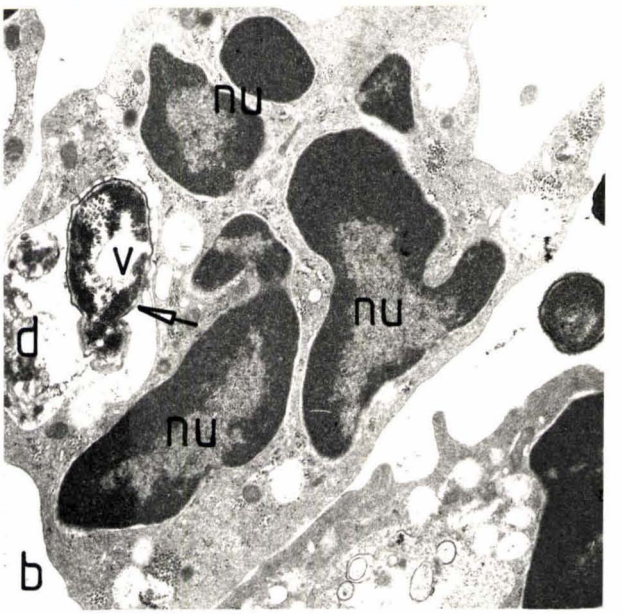
(b) A damaged bacterium can be seen in the phagosome of this neutrophil. The cell wall and membrane of the bacterium are damaged (arrow) and the cytoplasm is vacuolated (v). Debris (d) can also been seen in the phagosome.
x 14207.

(c) A neutrophil containing intact (i) and degraded (d) bacteria within phagosomes. Debris (arrowheads) can also be seen within the phagosomes.
x 17486.

nu = neutrophil nucleus

N.B. Neutrophils in this Figure are not labelled for peroxidase activity.


g
nu
nu
a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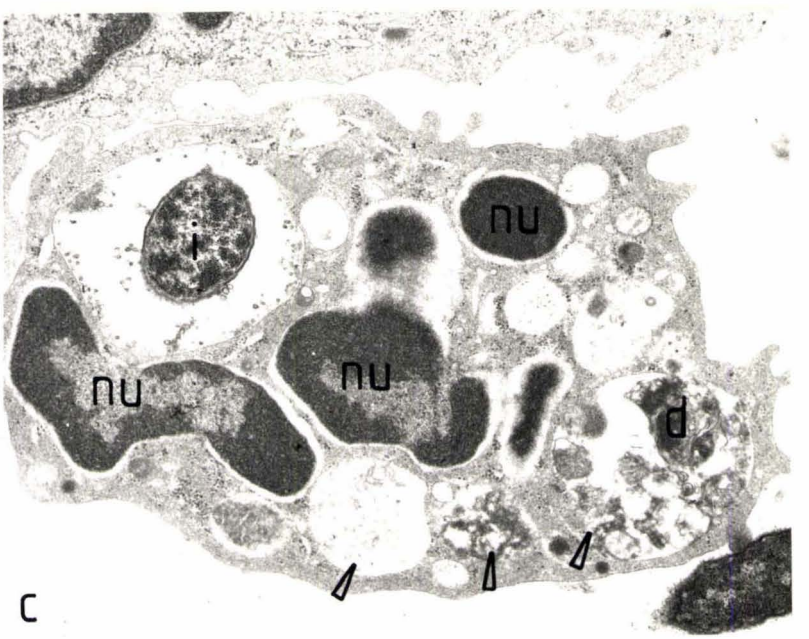
nu
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c

and lack of integrity of the cell wall and membrane. Some neutrophils contained both intact bacteria and the remnants of degraded bacteria. Fig.4.19c shows a neutrophil with a phagosome containing an intact bacterium, with evidence of degranulation, and phagosomes containing the end products of bacterial degradation.

The incubation of E. coli, pre-opsonized in 50% NS and 50% IS, with peritoneal leukocytes in 10% NS for 60 mins (Table 4.3) also led to more intracellular bacteria being seen in ultra-thin sections of neutrophils. Slightly damaged bacteria were seen in phagosomes (Fig.4.20a & b), as well as more severely damaged bacteria (Fig.4.20c & d) and bacterial debris (Fig.4.20e & f). Peroxidase-positive and peroxidase-negative granules were still visible in the neutrophils' cytoplasm but some reaction product for peroxidase can be seen in the phagosomes containing debris (Fig.4.20c-f).

4.2.5d Intracellular Killing of B. fragilis, B. vulgatus and E. coli in the Absence of On-going Phagocytosis

Ultrastructural studies on the fate of the intracellular bacteria observed during phagocytic killing assays showed the destruction of bacteria, in particular B. fragilis, was not prevalent (Sections 4.2.5b and c). Intracellular killing assays were used to measure the viability of a discrete load of intracellular bacteria over a 120 min period.

B. fragilis, B. vulgatus and E. coli, which had been pre-opsonized in 10% NS or 10% NS and 10% IS were ingested by $5x10^6$ peritoneal leukocytes over a 3 min period before the removal of extracellular bacteria by differential

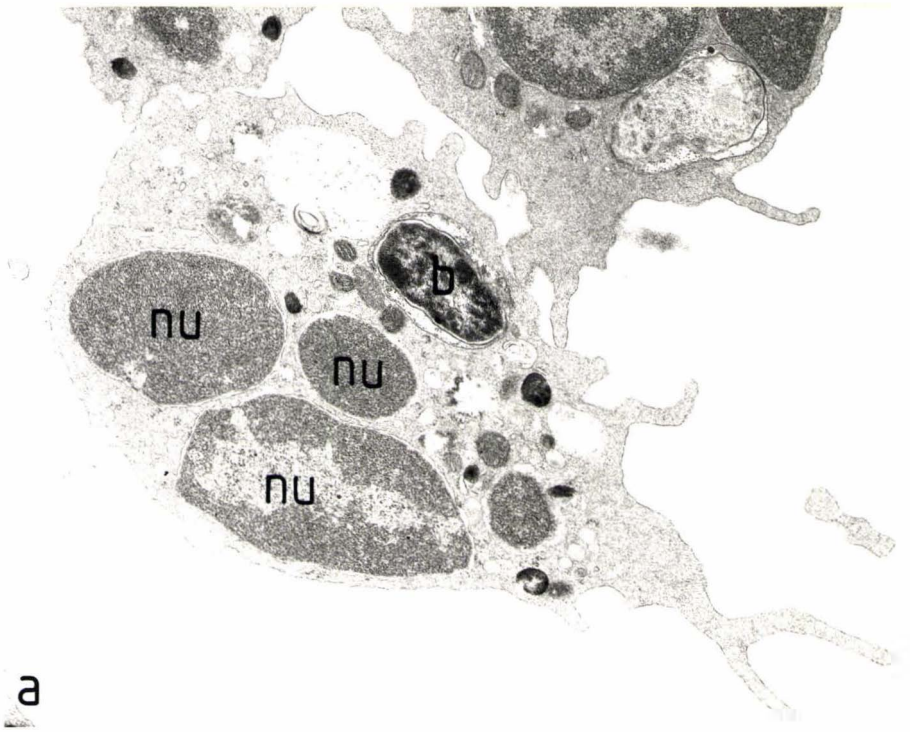
Figure 4.20: Phagocytosed *E. coli* (pre-opsonized in 50% NS and 50% IS) after 60 mins in 10% NS

(a) A neutrophil with a slightly damaged bacterium (b) in a phagosome. The bacterium (enlarged in (b)) has a disrupted cell wall (arrow). The phagosome is peroxidase-negative. Peroxidase-positive primary (pg) and peroxidase-negative secondary (sg) granules can be seen in the neutrophil's cytoplasm.
x 15360; (b) x 27257.

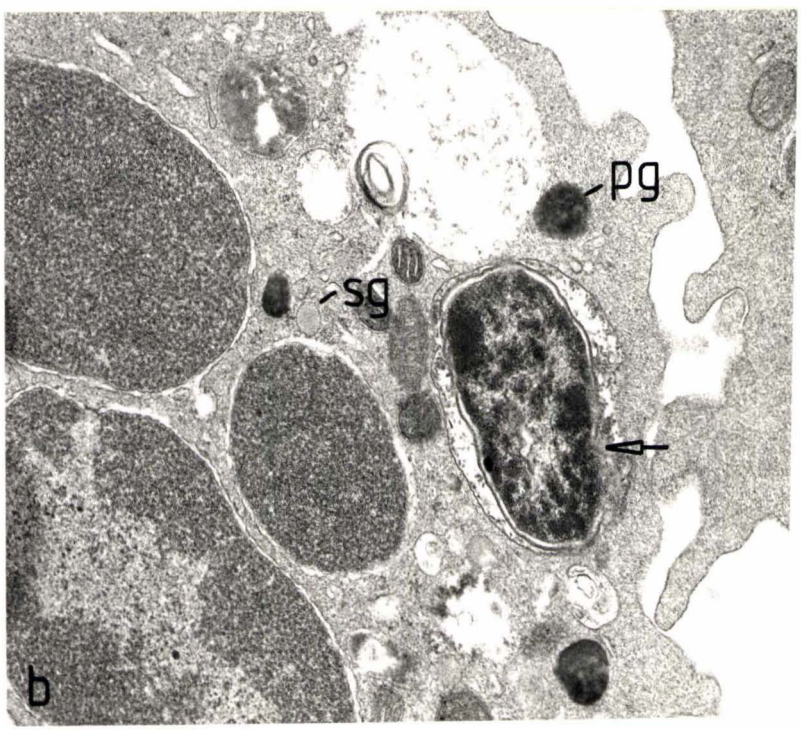
(c) A more severely damaged bacterium (b) can be seen in a phagosome of this neutrophil. The bacterium is enlarged in (d) and has a disrupted cell wall and membrane (arrow) and the bacterial cytoplasm has a reduced electron density. A peroxidase-positive phagosome (p) containing debris is also visible. Primary (pg) and secondary (sg) granules can be seen.
x 14400; (d) x 36343.

(e) A neutrophil with phagosomes containing bacterial debris (de), damaged (d) and intact (i) bacteria (enlarged in (f)). Peroxidase-positive material can be seen in some phagosomes (arrowheads). Primary (pg) and secondary (sg) granules are visible.
x 10029; (f) x 17923.

nu = neutrophil nucleus



a



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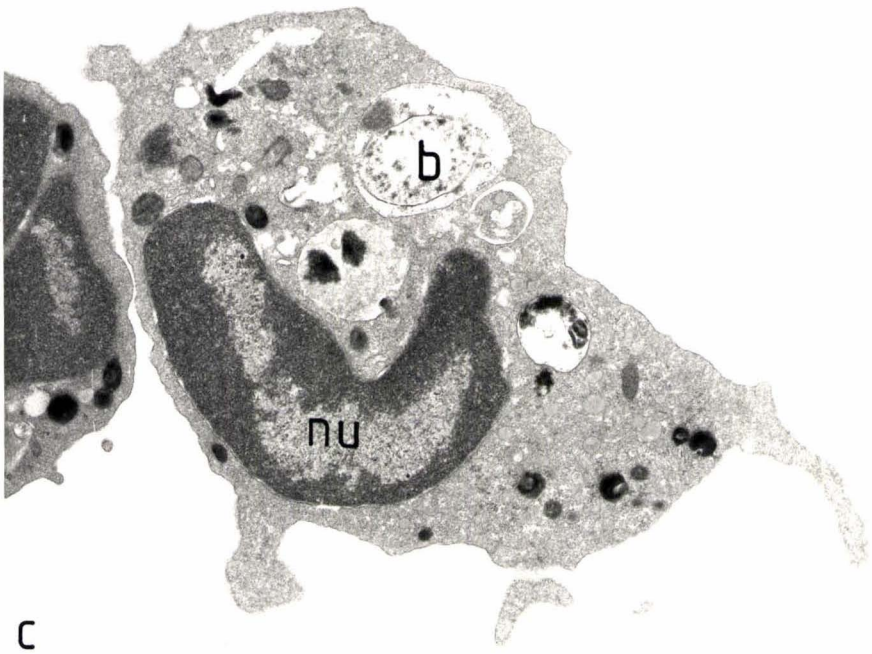

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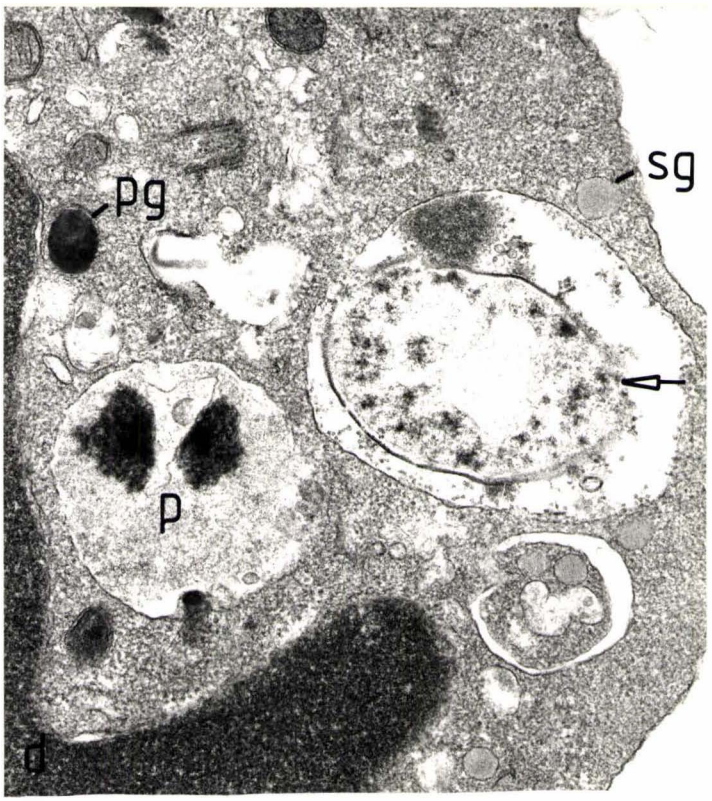
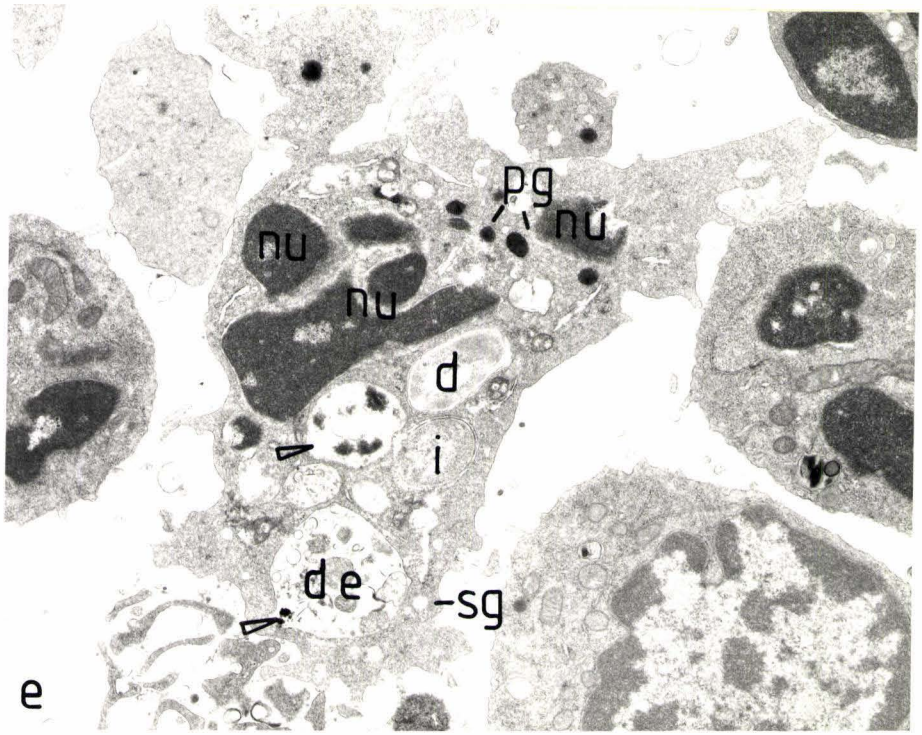


d


pg
nu
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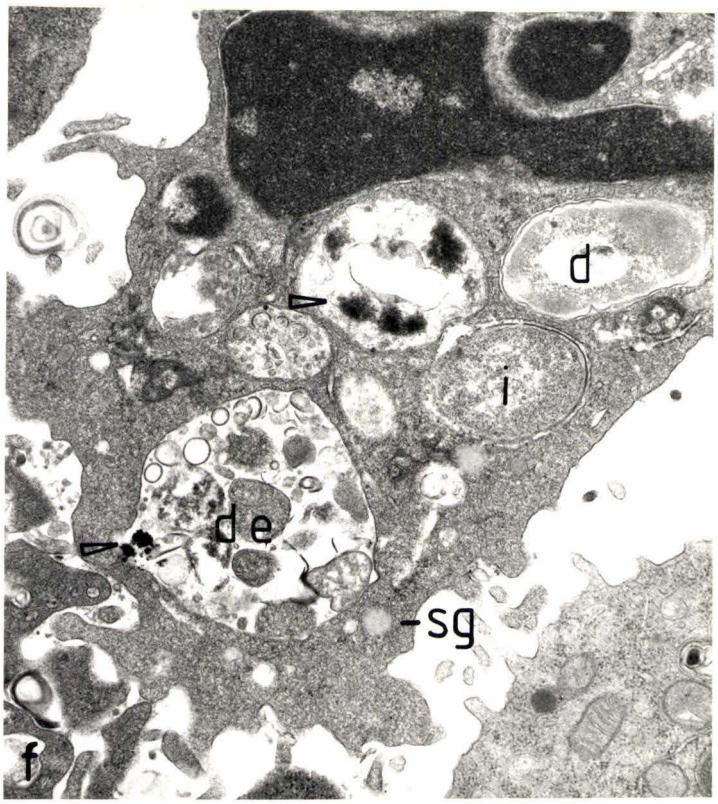
d
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centrifugation. The numbers of viable intracellular bacteria and the resulting ratios of viable intracellular bacteria to peritoneal leukocytes are shown in Table 4.8. In aerobic and anaerobic conditions significantly more B. fragilis were phagocytosed when pre-opsonized in 10% NS and 10% IS than in 10% NS ($p < 0.05$). Significantly more viable intracellular B. fragilis, which had been pre-opsonized in 10% NS and 10% IS, were detected in anaerobic conditions than in aerobic conditions ($p < 0.05$). When bacteria were pre-opsonized in 10% NS, fewer B. vulgatus than B. fragilis were ingested. However, more B. vulgatus than B. fragilis were phagocytosed when pre-opsonized in 10% NS and 10% IS. The pre-opsonization of E. coli in 10% NS and 10% IS did not result in significantly greater numbers of intracellular bacteria when compared with E. coli pre-opsonized in 10% NS.

In the absence of extracellular NS, only 4% and 21% of intracellular B. fragilis, pre-opsonized in either 10% NS or 10% NS and 10% IS respectively, were killed after 60 mins in aerobic conditions. However, in the presence of extracellular 10% NS, some killing of the ingested bacteria occurred in aerobic but not in anaerobic conditions (Table 4.9). After 60 mins in aerobic conditions, 62% of B. fragilis and 84% of B. vulgatus, both pre-opsonized in 10% NS, had been killed. When pre-opsonized in 10% NS and the appropriate IS, 71% of B. fragilis and 54% of B. vulgatus had been killed after 60 mins in aerobic conditions. Intracellular killing of pre-opsonized B. fragilis and B. vulgatus had not occurred after 120 mins in anaerobic conditions. Intracellular E. coli, which had been pre-opsonized in 10% NS or 10% NS and 10% IS, were killed to a minimal extent after 60 mins in aerobic conditions.

Table 4.8: Number of Viable Intracellular Bacteria after 3 mins of Phagocytosis

Bacteria	Pre-opsonizing Conditions	O_2	No. Viable Intracellular Bacteria	Ratio of Intracellular Bacteria to Peritoneal Leukocytes
B. fragilis	10% NS	+	$1.84 \pm 0.52 \times 10^6$ (2)[a]	1:2.7
		-	$2.59 \pm 0.93 \times 10^6$ (5)	1:1.9
	10% NS + 10% IS	+	$6.90 \pm 3.55 \times 10^6$ (14)	1.4:1
		-	$1.71 \pm 0.30 \times 10^7$ (5)	3.4:1
B. vulgatus	10% NS	+	$2.00 \pm 0.30 \times 10^5$ (2)	1:25
		-	7.30×10^5 (1)	1:6.8
	10% NS + 10% IS	+	$1.42 \pm 1.12 \times 10^7$ (2)	2.8:1
		-	5.30×10^7 (1)	10.6:1
E. coli	10% NS	+	$1.60 \pm 0.60 \times 10^5$ (3)	1:31
		-	ND[b]	ND
	10% NS + 10% IS	+	$1.40 \pm 0.80 \times 10^5$ (3)	1:36
		-	ND	ND

Peritoneal leukocytes (5×10^6) and 5×10^8 pre-opsonized bacteria in either RPMI or 1% NS were incubated for 3 mins at 37°C under rotation in aerobic and anaerobic conditions before the removal of extracellular bacteria by differential centrifugation. The numbers of ingested bacteria were assessed by viable counts

[a] Figures in brackets represent the number of experiments
[b] ND = not done

Table 4.9: Intracellular Killing of *B. fragilis*, *B. vulgatus* and *E. coli*

Bacteria	Pre-opsonizing Conditions	Sampling Time	Intracellular Killing Aerobic		Anaerobic
B. fragilis	10% NS	60 mins	-0.42 ± 0.06[a]	(62%)[b]	0.04 ± 0.15
		120 mins	ND[c]		0.06 ± 0.24
	10% NS + 10% IS	60 mins	-0.54 ± 0.34	(71%)	-0.06 ± 0.08
		120 mins	-0.63 ± 0.26	(77%)	0.20 ± 0.29
B. vulgatus	10% NS	60 mins	-0.79 ± 0.14	(84%)	0.03
		120 mins	ND		-0.06
	10% NS + 10% IS	60 mins	-0.34 ± 0.25	(54%)	-0.11
		120 mins	ND		0.04
E. coli	10% NS	60 mins	0.11 ± 0.17		ND
		120 mins	ND		ND
	10% NS + 10% IS	60 mins	-0.11 ± 0.11	(22%)	ND
		120 mins	ND		ND

Peritoneal leukocytes ($5x10^6$) and $5x10^8$ pre-opsonized bacteria in RPMI or 1% NS were incubated for 3 mins at 37°C, in aerobic and anaerobic conditions under rotation, before the removal of extracellular bacteria by differential centrifugation. The peritoneal leukocytes were re-suspended in 10% NS and re-incubated at 37°C under rotation. The viability of the ingested bacteria was measured after a further 60 and 120 mins in aerobic and anaerobic conditions

[a] Change in the viability ($\log_{10}$ cfu/ml) of bacteria : mean $\pm$ 1 SD of either 2-6 (*B. fragilis*), 1-2 (*B. vulgatus*) or 3 (*E. coli*) experiments

[b] Figures in brackets represent % bacteria killed

[c] ND = not done

Electron microscopy was used to observe B. fragilis (pre-opsonized in 10% NS and 10% IS) within neutrophils 60 mins after the short time allowed for ingestion of the bacteria. In aerobic conditions, in the absence of on-going phagocytosis, intracellular bacteria were seen in a variety of states within phagosomes of neutrophils. Bacteria showing varying degrees of damage were sometimes seen within the same phagosome of a neutrophil (Fig.4.21a). Widespread bacterial degradation within a neutrophil's phagosomes is evident in Fig.4.21b & c. Vacuolation of the bacterial cytoplasm and breakdown of the cell walls and membranes of the bacteria can be seen.

The effect of heat-killed E. coli on the intracellular killing of B. fragilis by peritoneal leukocytes was tested in aerobic conditions (Table 4.10). Pre-opsonized B. fragilis were ingested by peritoneal leukocytes for 3 mins before removal of extracellular bacteria by differential centrifugation. The peritoneal leukocytes were re-suspended in 10% NS plus killed E. coli and the intracellular killing of B. fragilis was measured. There were no significant differences in the intracellular killing of B. fragilis in the presence or absence of varying concentrations of killed E. coli. In a similar experiment performed in anaerobic conditions, the presence of killed or live E. coli did not affect the intracellular killing of B. fragilis (Table 4.11). There was an increase in the number of viable E. coli added over the 120 min assay period.

Figure 4.21: Intracellular pre-opsonized B. fragilis after 60 mins in 10% NS in the absence of on-going phagocytosis

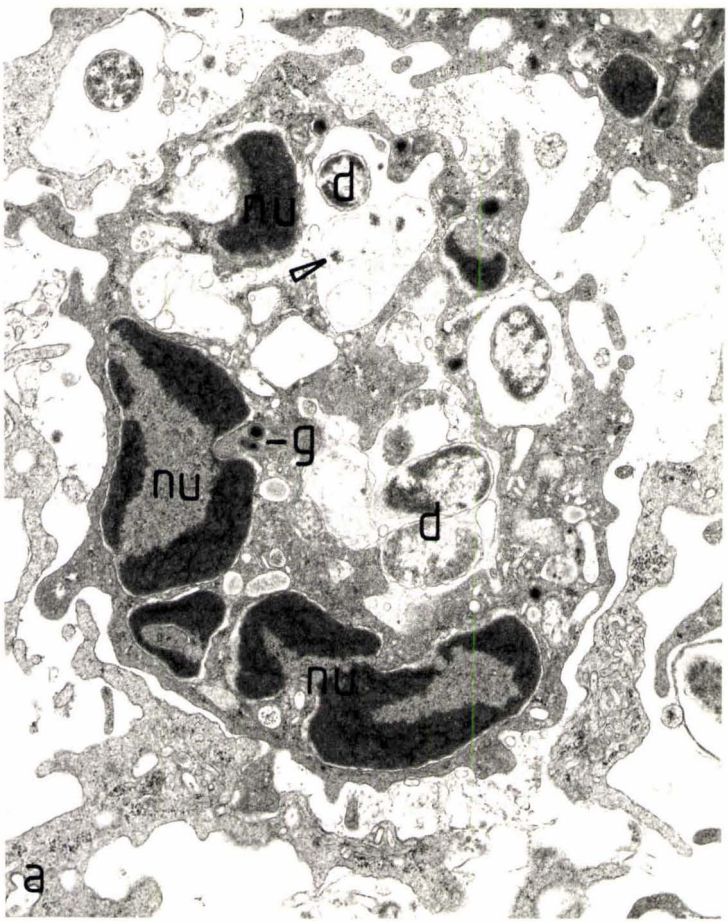
(a) A neutrophil containing damaged (d) bacteria within phagosomes. Bacterial debris, e.g. remnants of cell walls (arrow), is also visible in the phagosomes. Granules (g) can be seen in the neutrophil's cytoplasm.
x 14400.

(b) Part of a neutrophil with large phagosomes (p1 and p2) containing bacteria (b) showing varying amounts of damage.
x 18579.

c) Part of phagosome p1 in (b) to show the stages of bacterial destruction. Increasing vacuolation of the bacterial cytoplasm ($^{V}1$ to ^{V}n) is accompanied by the breakdown of the bacterial cell walls and membranes (arrows).
x 32709.

nu = neutrophil nucleus

N.B. The neutrophils in this Figure were not labelled for peroxidase activity.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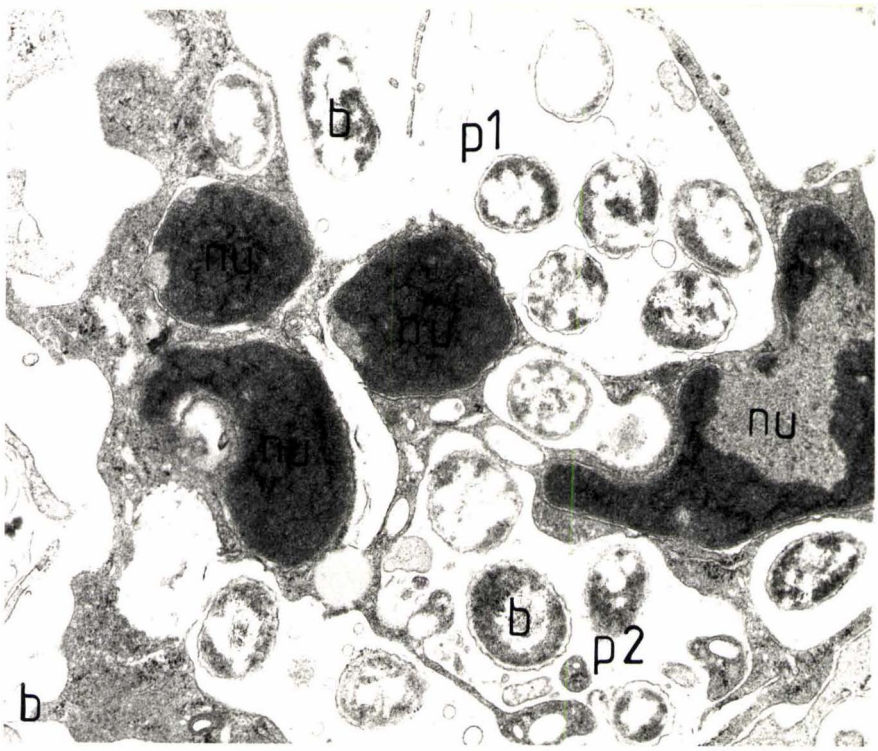

b
p1
nu
nu
nu
nu
b
p2
b

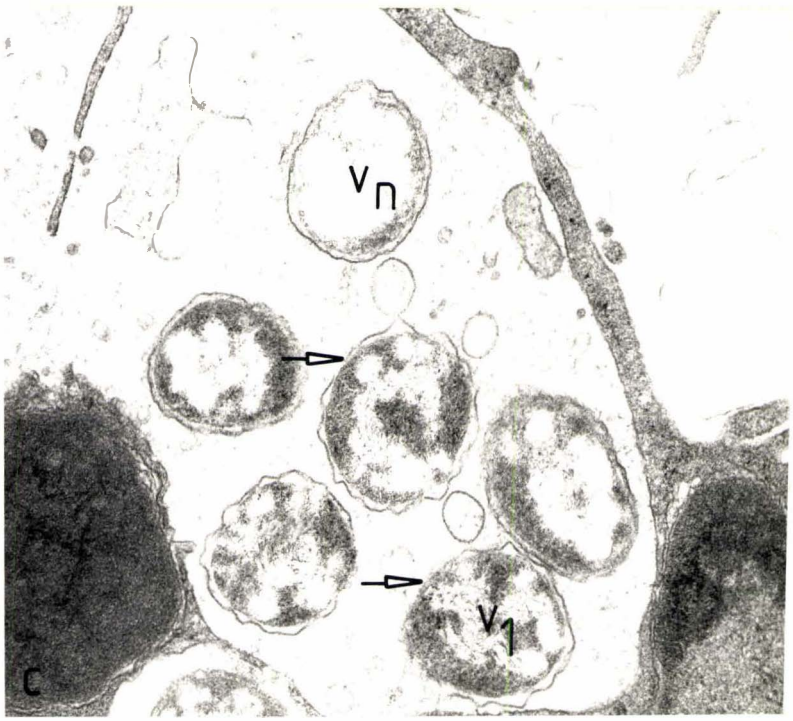

v_n
v_1
c

Table 4.10: The Effect of Killed *E. coli* on the Intracellular Killing of Pre-opsonized *B. fragilis* in Aerobic Conditions

Sampling Time	Intracellular Killing in the Presence of				
	No *E. coli*	$5x10^5$ *E. coli*	$5x10^6$ *E. coli*	$5x10^7$ *E. coli*	$5x10^8$ *E. coli*
60 mins	-0.54 ± 0.34[a]	-0.72 ± 0.31	-0.95 ± 0.54	-0.55 ± 0.42	-0.78 ± 0.36
120 mins	-0.63 ± 0.26	-0.68 ± 0.02	-0.95 ± 0.56	-0.66 ± 0.20	-0.98 ± 0.22

B. fragilis ($5x10^8$), pre-opsonized in 10% NS and 10% IS, were phagocytosed in the presence of 1% NS. Extracellular bacteria were removed before the addition of 10% NS and killed *E. coli* to the peritoneal leukocytes.

[a] Change in the viability ($\log_{10}$ cfu/ml) of *B. fragilis* after 60 and 120 mins : mean $\pm$ 1 SD of two experiments

Table 4.11: The Effect of *E. coli* on the Intracellular Killing of Pre-opsonized *B. fragilis* in Anaerobic Conditions

B. fragilis pre-opsonized in:	Sampling Time	Intracellular Killing in the Presence of No *E. coli*	10^6 live *E. coli*	10^6 killed *E. coli*	$5x10^8$ killed *E. coli*
10% NS	60 mins	0.04 ± 0.15[a]	-0.08	-0.13	-0.08
	120 mins	0.06 ± 0.24	0.04	0.10	0.34
10% NS + 10% IS	60 mins	-0.06 ± 0.08	-0.02	-0.02	-0.34
	120 mins	0.20 ± 0.29	0.40	0.35	0.21

B. fragilis ($5x10^8$), pre-opsonized in 10% NS or 10% NS and 10% IS, were phagocytosed in the presence of 1% NS. Extracellular bacteria were removed before addition of 10% NS and *E. coli* to the peritoneal leukocytes

[a] Change in the viability ($\log_{10}$ cfu/ml) of *B. fragilis* after 60 and 120 mins

4.2.6 The Effect of Bran on the Phagocytic Killing of B. fragilis and E. coli

In the presence of bran, leukocytes were clumped *in vitro* (Fig.4.22). Bran was phagocytosed by neutrophils *in vitro* (Fig.4.23a) and was seen in the same phagosomes as bacteria (Fig.4.23b).

The effect of bran on killing by peritoneal leukocytes was measured using pre-opsonized *B. fragilis* and *E. coli*. *B. fragilis* was pre-opsonized in 10% NS and 10% IS; *E. coli* was pre-opsonized in 50% NS and 50% IS. Pre-treatment of $5x10^6$ peritoneal leukocytes with 1 mg of bran, for 30 mins at 37°C, prior to the addition of $5x10^5$ pre-opsonized *B. fragilis* and 2% NS, did not affect the phagocytic killing of the bacteria. The decrease in the viability of *B. fragilis* was 1.3 logs at 60 mins and 1.9 logs by 120 mins.

Fig.4.24 shows the effect of bran on the phagocytic killing of *B. fragilis*. There were no significant differences in the phagocytic killing of *B. fragilis* at 60 and 120 mins in the presence of 10% NS compared with 2% NS. However, bran significantly reduced phagocytic killing in the presence of 10% NS ($p < 0.05$) but not in the presence of 2% NS at 60 and 120 mins. A significant reduction in the phagocytic killing of *B. fragilis* was also observed in the presence of 10% NS and 1 mg of bran which had been pre-opsonized in 10% NS ($p < 0.05$).

Reductions in the numbers of pre-opsonized *E. coli* in the presence of peritoneal leukocytes, serum and bran occurred in the first 60 mins of the phagocytic killing assays (Fig.4.25). By 120 mins growth of *E. coli* had occurred. However, statistically significant differences were apparent at 60 and 120 mins. The numbers of surviving pre-opsonized *E. coli*, in the presence of peritoneal leukocytes, were

Figure 4.22: The clumping of leukocytes in the presence of bran. Peritoneal leukocytes ($5x10^6$) were incubated with 1 mg of bran (Br) in 10% NS for 20 mins. Neutrophils (pmn) and mononuclear cells (mn) are visible.
x 1250.

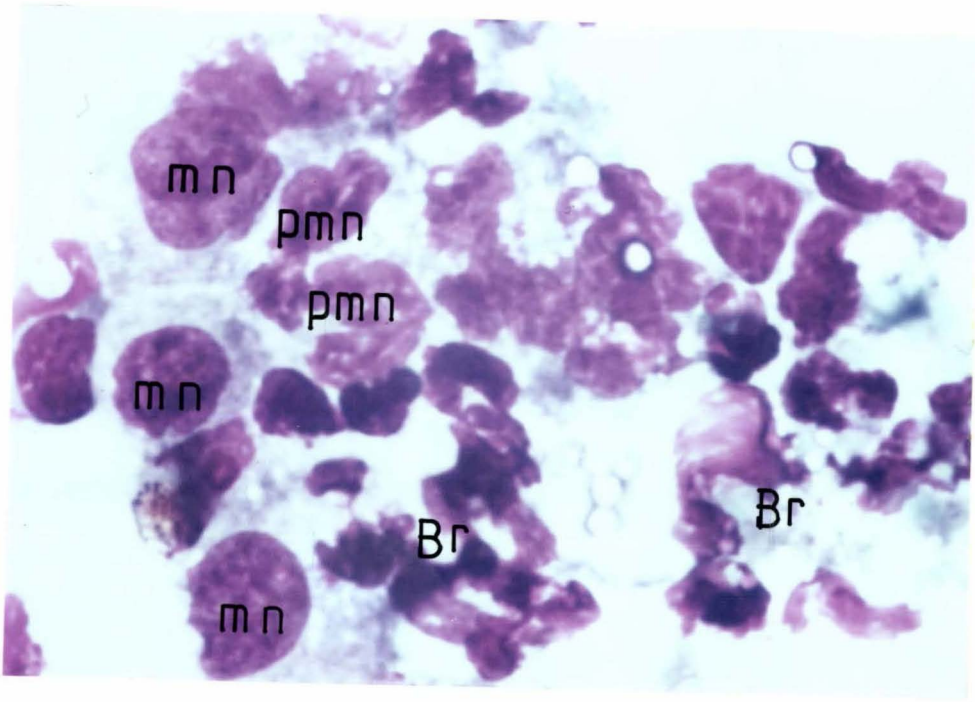
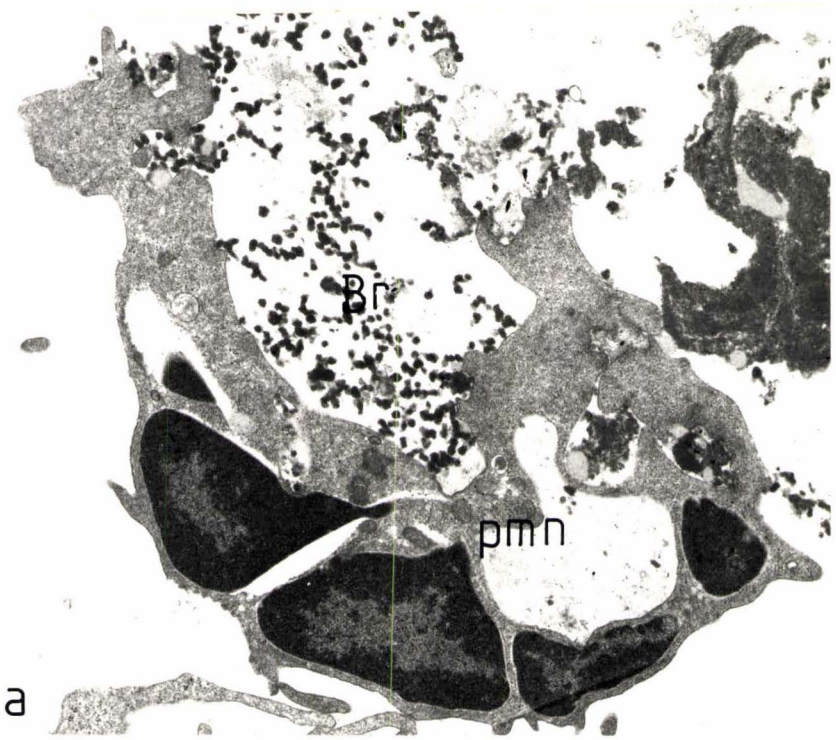

mn
pmn
pmn
mn
Br
Br
mn

Figure 4.23: The phagocytosis of bran by peritoneal neutrophils after 20 mins in 10% NS

(a) A neutrophil (pmn) phagocytosing bran (Br). x 13600.

(b) The large phagosome of this neutrophil contains both bran (Br) and bacteria (arrows). x 14400.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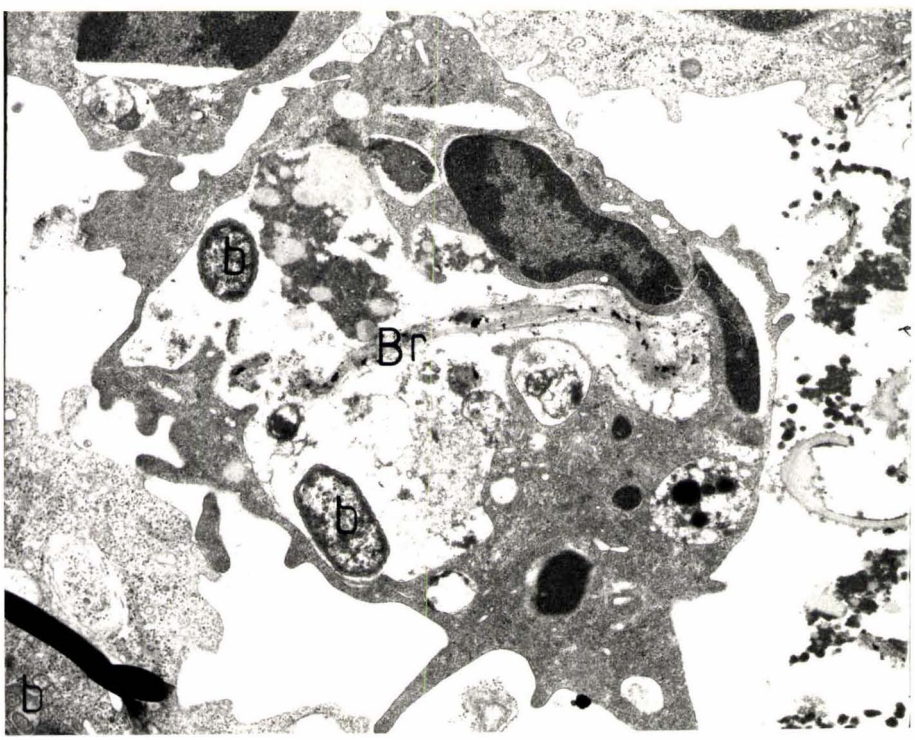
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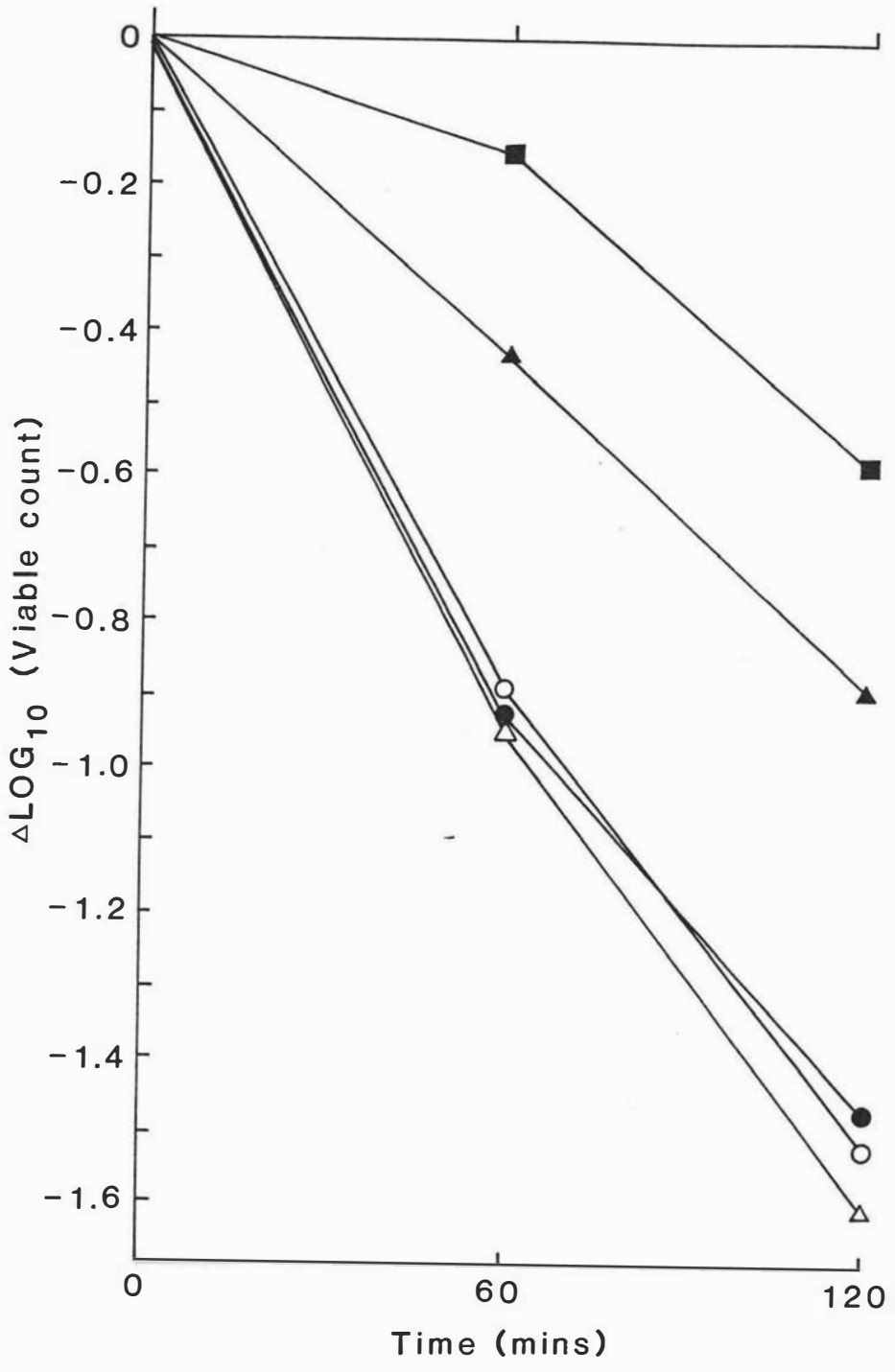

Figure 4.24: The effect of bran on the phagocytic killing of B. fragilis in aerobic conditions. Peritoneal leukocytes ($5x10^6$) were incubated with $5x10^5$ pre-opsonized B. fragilis in either 2% NS (○), 2% NS and 1 mg bran (●), 10% NS (△), 10% NS and 1 mg bran (▲), or 10% NS and 1 mg bran pre-opsonized in 10% NS (■) at 37°C under rotation

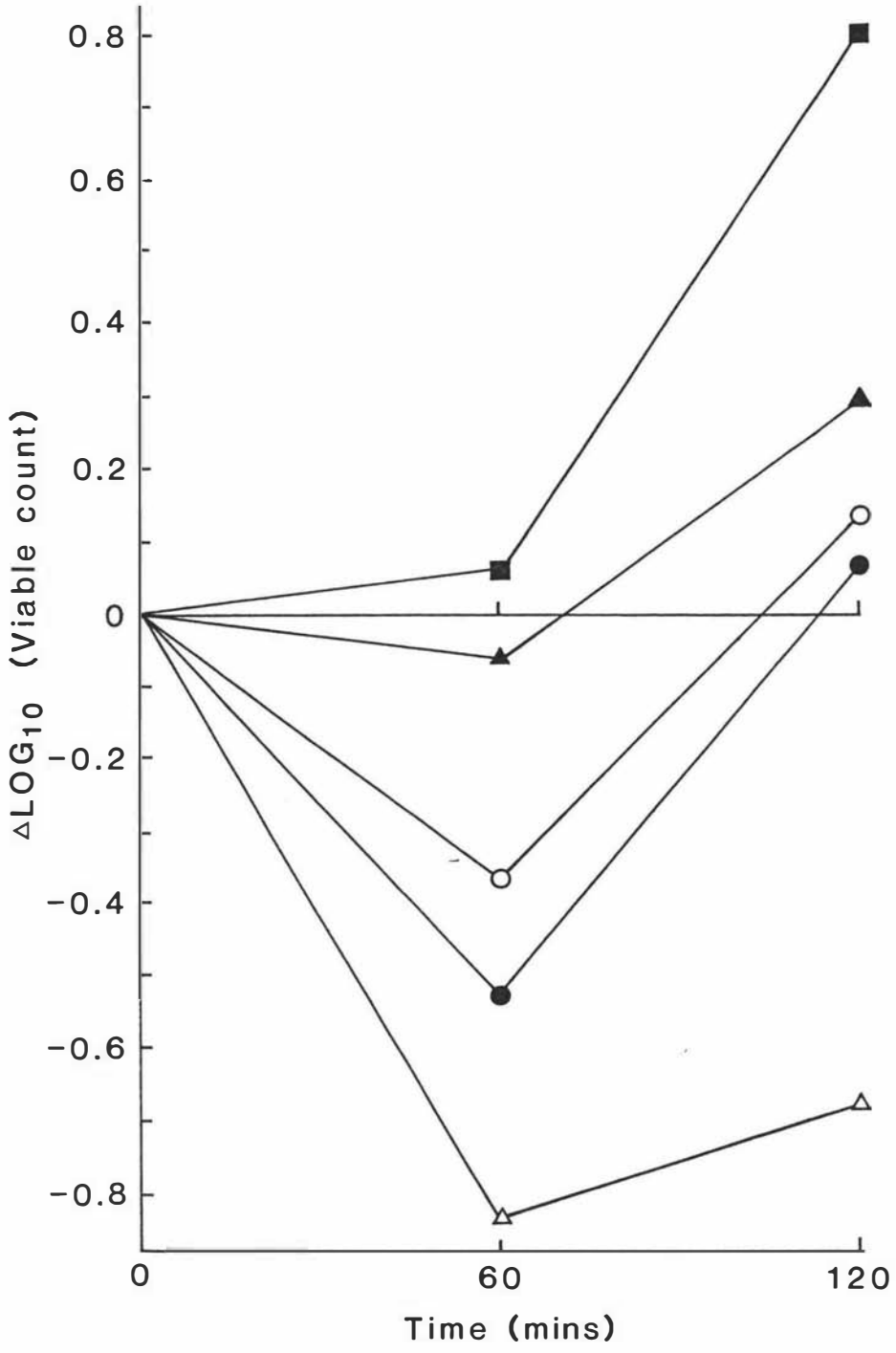

Figure 4.25: The effect of bran on the phagocytic killing of E. coli in aerobic conditions. Peritoneal leukocytes ($5x10^6$) were incubated with $5x10^5$ pre-opsonized E. coli in either 2% NS (o), 2% NS and 1 mg bran (●), 10% NS (△), 10% NS and 1 mg bran (▲) or 10% NS and 1 mg bran pre-opsonized in 10% NS (■) at 37°C under rotation

significantly less in 10% NS than in 2% NS at 60 and 120 mins ($p < 0.05$). The presence of bran led to a significant increase in the viable count of *E. coli* in 10% NS with peritoneal leukocytes ($p < 0.05$) but not in 2% NS plus peritoneal leukocytes. Significantly more *E. coli* survived in the presence of peritoneal leukocytes, 10% NS and 1 mg of bran which had been pre-opsonized in 10% NS ($p < 0.05$).

4.3 DISCUSSION

4.3.1 Phagocytic Killing of Logarithmic and Stationary Phase Bacteria

It has been shown that bacteria exhibit slow growth rates *in vivo*. Rapidly growing, logarithmic phase bacteria differ from stationary phase bacteria in many properties that influence survival *in vivo*. These differences can be attributed to the availability of nutrients and other environmental factors which also affect other properties of the bacteria, including the structure and function of the bacterial surface (Brown & Williams, 1985). In turn, the nature of the bacterial surface can contribute to the virulence of an organism, as was demonstrated by Simon *et al*. (1982). They observed more capsular material around animal-passaged isolates of *B. fragilis* than around laboratory-passaged isolates. Animal-passaged *B. fragilis* were more resistant to opsonization and phagocytic killing by human neutrophils. However, Pruul *et al*. (1983) found clinical isolates and logarithmic phase cultures of clinical isolates of *B. fragilis* to be equally susceptible to the bactericidal mechanisms of neutrophils. A later study showed stationary phase cultures of a clinical isolate of *B. fragilis* were less susceptible to killing by neutrophils than were logarithmic phase cultures of the same clinical isolate (Wetherall *et al*., 1984).

Thus, although no statistically significant differences in the phagocytic killing of logarithmic and stationary phase cultures of B. fragilis were found in the present study (Table 4.1), stationary phase cultures were used for in vitro experiments because stationary phase bacteria were used in AIM.

4.3.2 Phagocytic and Intracellular Killing of B. fragilis, B. vulgatus and E. coli

The ability of B. fragilis and E. coli to persist in IA abscesses despite the presence of large numbers of phagocytic cells, especially neutrophils, suggests that the requirements for opsonization and/or killing by the phagocytes are not being met. A mixture of leukocyte types is found in the stimulated and unstimulated murine peritoneal cavity (Daems, 1980) and in the initiation of IA abscess development in mice (Table 3.4). In addition, it is recognized that neutrophils and macrophages do not function independently of one another (e.g. Ishibashi & Yamashita, 1987; Koivuranta-Vaara et al., 1987; Peveri et al., 1988). Thus, mixtures of peritoneal leukocytes (Section 2.11) were used in the present in vitro studies of phagocytosis and intracellular killing.

Both B. fragilis and B. vulgatus were readily phagocytosed by murine peritoneal neutrophils in NS or NS and IS in vitro (Table 4.2). Although the cytocentrifuge smears were prepared without the removal of extracellular bacteria, the neutrophils appeared to have ingested more Bacteroides than E. coli. The clumps of apparently intracellular bacteria were confined by the neutrophils' cell membranes (Fig.4.1). Intracellular Bacteroides, especially B. fragilis, were too numerous to be counted, although Leijh et al. (1979b), working with S. aureus and E. coli, calculated that between 40-50 bacteria can be phagocytosed by a single human

neutrophil. Neutrophils outnumbered macrophages and monocytes in the populations of leukocytes obtained by the peritoneal lavage of mice (Section 2.11), but there was some participation of mononuclear phagocytes in the phagocytosis of Bacteroides species and E. coli in the assay system. A lack of involvement of mononuclear phagocytes in the phagocytosis and killing of Proteus mirabilis has been reported (Finlay-Jones et al., 1984). Mononuclear phagocytes are able to phagocytose in suspension (Leijh et al., 1979b) but neutrophils are more aggressively phagocytic than mononuclear cells (Horwitz, 1982).

Both strains of B. fragilis and B. vulgatus used in this study possess a capsule as demonstrated by electron microscopy (Fig.3.1a, b & e). The susceptibility of these encapsulated strains to phagocytosis is in agreement with the results of Tofte et al. (1980), Ellis and Barrett (1982) and Bjornson et al. (1983). In contrast, others have suggested that the capsule of B. fragilis is an important virulence factor (Kasper et al., 1977; Onderdonk et al., 1977) and responsible for resistance to phagocytosis and killing by neutrophils (Simon et al., 1982). It has been suggested that the density and arrangement of capsular material may determine resistance to phagocytosis rather than just the presence of a capsule (Bjornson, 1984). This may explain why the encapsulated strains of B. fragilis MFN 1110 and B. vulgatus MFN 15 used in the present study were readily phagocytosed in the absence of detectable antibody.

E. coli showed resistance to phagocytosis (Table 4.2, Table 4.3, Fig.4.1c) which has been previously documented (Medearis et al., 1968; Howard & Glynn, 1971; Rozenberg-Arska et al., 1986; Allen et al., 1987). The resistance to phagocytosis has been attributed to the possession of capsular (K) antigens by some strains of E. coli (Howard & Glynn, 1971; Rozenberg-Arska et al., 1986; Allen et al., 1987).

Rozenberg-Arska et al. (1986) demonstrated loss of resistance to phagocytosis of an encapsulated E. coli strain when the K antigens of the strain were destroyed by heating. When the strain of E. coli used in the present study was heated, there was more extensive phagocytosis of the organism by murine peritoneal leukocytes (Fig.4.2). The capsule of E. coli K1 studied by Allen et al. (1987) is poorly immunogenic and in the present study, high titre immune serum was not obtained following the immunization of mice with E. coli (Section 2.8). This may explain why the phagocytosis of E. coli in NS and IS was similar to that in NS alone. The serotype of the strain of E. coli used in the present study is unknown and an effect of K antigens on phagocytosis cannot be discounted. The organism does possess a thin layer of material external to the cell wall when observed by electron microscopy (Fig.3.1f), which may contribute to the antiphagocytic nature of this strain of E. coli.

The phagocytic killing of B. fragilis, B. vulgatus and E. coli was determined at a ratio of one bacterium per ten peritoneal leukocytes, so differences in serum requirements could be demonstrated. Both complement and antibodies to B. fragilis were required for maximal phagocytic killing of B. fragilis by murine peritoneal leukocytes (Fig.4.3a). This is consistent with data from other groups using either human (Bjornson & Bjornson, 1978; Tofte et al., 1980) or mouse (Ellis & Barrett, 1982; Nulsen, 1982) assay systems.

Human NS contains antibodies to B. fragilis (Quick et al., 1972) but although antibodies to a murine strain of Bacteroides have been detected in murine NS at low titres (Foo & Lee, 1972), NS from C3H mice used in this study did not contain detectable antibody to B. fragilis (Section 2.8). This may explain the lack of killing of B. fragilis in C3H mouse NS alone, although the organisms were phagocytosed (Table 4.2).

The lack of killing of B. fragilis by peritoneal leukocytes in C3H mouse NS (Fig.4.3a) may also be due to a deficiency in the complement system. The mouse complement system is known to differ from other animal complement systems in terms of biochemistry and function (Klaus et al., 1979; Sassi et al., 1987) and differences in complement activity among strains of mice have been documented (Terry et al., 1963). Finlay-Jones (personal communication) studied the phagocytic killing of E. coli in assay systems using combinations of peritoneal leukocytes and sera from Balb/c and C3H mice. Phagocytic killing by C3H or Balb/c leukocytes was reduced in C3H NS when compared to Balb/c NS. This indicates differences exist between Balb/c and C3H NS, probably due to differences in complement activity.

Specific antiserum was not necessary for the killing of B. vulgatus by murine peritoneal leukocytes (Fig.4.3b). Thus, the strains of B. fragilis and B. vulgatus have different serum requirements for phagocytic killing. This correlates with in vivo observations on the inability of the B. vulgatus strain to persist in abscesses when compared to the B. fragilis strain (Section 3.2.2). However, a difference in susceptibility to phagocytic killing of these species may not be the sole explanation for the overall greater virulence of B. fragilis in the murine model of IA abscess formation, since Vel et al. (1986) found no evidence to suggest that strains of B. fragilis were more capable of surviving phagocytic killing than were strains of B. vulgatus. In addition, Pruul et al. (1983) found a human neutrophil granule extract to be bactericidal to all strains of Bacteroides species tested, including B. fragilis and B. vulgatus.

In vitro, maximum killing of E. coli by murine peritoneal leukocytes required NS and IS (Fig.4.3c). The requirement

was probably due to the bacterial capsule. Nulsen (1982) also demonstrated more phagocytic killing of E. coli by Balb/c murine peritoneal leukocytes in NS and IS. Other in vitro studies using human peripheral blood leukocytes have shown that some strains of E. coli require both the complement and antibody components of human NS for maximum phagocytic killing (Guckian et al., 1978; Leist-Welsh & Bjornson, 1979; Horwitz & Silverstein, 1980). In the study of Horwitz & Silverstein (1980), the strains of E. coli requiring antibody for phagocytic killing pussessed a capsule.

Thus B. fragilis and E. coli, which form the most virulent combination in AIM in vivo (Table 3.1, Fig.3.2), both require complement and specific antibody for maximum killing by murine peritoneal leukocytes to occur in vitro.

Neutrophils possess oxygen-dependent and oxygen-independent microbicidal mechanisms and the ability of neutrophils to kill some bacteria can vary in aerobic and anaerobic conditions (Mandell, 1974; Ingham et al., 1981; Vel et al., 1984). In this study there was a significant reduction in the phagocytic killing of B. fragilis and E. coli in anaerobic conditions (Table 4.4). The reduced phagocytic killing of E. coli in anaerobic conditions vs aerobic conditions is in agreement with Mandell (1974) but not Vel et al. (1984). Mandell (1974) found B. fragilis to be killed equally as well in aerobic and anaerobic conditions, and more than a one log decrease in the numbers of B. fragilis was achieved in the anaerobic phagocytic killing assay used by Bjornson and Bjornson (1978). The disparity in results may be due to differences in strains of B. fragilis and also Mandell (1974) and Bjornson & Bjornson (1978) both used human leukocytes and serum. In the present study, an inability of leukocytes to kill B. fragilis and E. coli in anaerobic conditions in vitro would indicate neutrophil bactericidal

mechanisms may be adversely affected in the anaerobic environment of an abscess (Hays & Mandell, 1974).

Many studies use low ratios of bacteria to phagocytes because it is possible to demonstrate optimal levels of phagocytic killing. However, injury to the human colon, which may contain up to 10^{12} bacteria per gram of contents, can result in infection of the peritoneal cavity with a large number of bacteria (Bartlett, 1981). When a concentration of $5x10^8$ B. fragilis, the numerically dominant bacterial species in AIM, was used for in vitro studies of phagocytic killing, it was evident that more bacteria were killed at a ratio of 100 bacteria per leukocyte than at a ratio of one bacterium per ten leukocytes (Table 4.5). However, Clawson and Repine (1976) considered the efficiency of bactericidal function to be an important parameter and defined it as the percentage of the bacteria eliminated by the neutrophils. Thus, in the present study, the proportion of bacteria killed was reduced at the high ratio of bacteria to peritoneal leukocytes (Table 4.5), despite avid phagocytosis of the bacteria in aerobic and anaerobic conditions (Table 4.2). IS was necessary for the phagocytic killing of B. fragilis at a low ratio of bacteria to leukocytes (Fig.4.3a). However, increasing the concentration of antibodies from 10% to 40% did not increase the ability of the peritoneal leukocytes to kill at a high ratio of bacteria to leukocytes. In addition, when results were expressed as the change in the viability ($\log_{10}$ cfu/ml) of the bacteria, significantly reduced phagocytic killing at a high concentration of B. fragilis (Fig.4.4) and E. coli (Fig.4.5) was observed.

Limitations to the bactericidal capability but not to the phagocytic ability of neutrophils have been reported (Simmons & Karnovsky, 1973; Clawson & Repine, 1976; Matheisz & Allen, 1979). Clawson & Repine (1976) found that at ratios greater than ten S. aureus per neutrophil, more phagocytosis than

intracellular killing of the bacteria occurred. At a ratio of 100 S. aureus per neutrophil, the ability of the neutrophils to kill had further declined, and the neutrophils' capacities for phagocytosis were saturated. Wetherall et al. (1984) showed that an extract of granule proteins from human neutrophils, tested in anaerobic conditions, had greatest activity against low numbers of B. fragilis. Thus, results indicate that at a high ratio of bacteria to leukocytes, the bactericidal functions do not proceed at the same rate at which the bacteria are phagocytosed by leukocytes (Clawson & Repine, 1976; Section 4.2.2; Section 4.2.5a). The ability of the neutrophils to kill would be further affected in the anaerobic environment of an abscess (Hays & Mandell, 1974) because, although phagocytosis was not reduced in anaerobic conditions (Table 4.2), phagocytic killing was decreased (Table 4.4).

In this murine model of IA abscess formation, bran was used to enhance the virulence of B. fragilis and E. coli. Bran is composed of polysaccharide-containing fibre (Saunders, 1978). Polysaccharides can activate complement via the alternative pathway (Marcus et al., 1971; Mergenhagen et al., 1973; Fearon & Austen, 1980) and Nulsen (1982) showed that both autoclaved caecal and colonic contents and bran inhibited the phagocytic killing of P. mirabilis by murine peritoneal leukocytes in the presence of NS. The inhibitory effect was due to interference of the potentiating agents with the alternative complement pathway-mediated opsonization of P. mirabilis. Another infection-potentiating agent, tryptic mucin, also has an anti-complementary action due to the polysaccharide, heparin, which it contains (Lambert & Richley, 1952).

In the present study, bran inhibited the phagocytic killing of B. fragilis and E. coli by murine peritoneal leukocytes in NS (Figs.4.24 and 4.25). This was due to an effect on serum

since pre-incubation of the peritoneal leukocytes with bran did not reduce killing. The bran was not simply competing with the bacteria for opsonins in NS since the bacteria were opsonized prior to their inclusion in the assays. Inhibition of phagocytic killing by bran was observed in 10% NS but not in 2% NS, implying the effect of bran was dependent not only on the presence of serum, but on the concentration of serum. Bran can be phagocytosed in 10% NS and was visible in the phagosomes of neutrophils (Fig.4.23).

Thus, bran may affect the intracellular killing mechanisms of neutrophils by itself being opsonized and phagocytosed and/or by interfering with the components of serum, probably complement, necessary for the stimulation of intracellular killing. Extracellular complement is necessary for the optimal intracellular killing of S. aureus and E. coli by human monocytes (Leijh et al., 1979a) and human neutrophils (Leijh et al., 1981), and of P. mirabilis by murine peritoneal neutrophils (Hart et al., 1985). Bran may also contribute to the localization of bacteria in the murine peritoneal cavity akin to the clumping factor of S. aureus (Kapral et al., 1980). Light microscopy (Fig.4.22) showed extensive clumping of murine peritoneal leukocytes occurred in the presence of bran in vitro which could assist abscess formation.

The interaction of bacteria with phagocytes involves opsonization and attachment of the organism to the phagocyte, ingestion and intracellular killing of the organism. In order to analyse intracellular killing as a process separate from phagocytosis, intracellular killing assays were undertaken. In extracellular NS, in aerobic conditions, a proportion of B. fragilis and B. vulgatus were killed within murine peritoneal leukocytes (Table 4.9), whereas negligible killing of intracellular B. fragilis was observed in the absence of extracellular NS. These results are consistent

with other studies which have shown extracellular NS is necessary for the stimulation of intracellular killing (Leijh et al., 1979a; Leijh et al., 1981; Hart et al., 1985). Despite the low ratio of intracellular E. coli to the peritoneal leukocytes (Table 4.8), the E. coli were not killed to any significant extent in aerobic conditions (Table 4.9). Both Bacteroides species survived in anaerobic conditions, which suggests the oxygen-independent mechanisms of the neutrophils were unable to kill the bacteria.

The loads of intracellular bacteria were obtained by allowing phagocytosis of bacteria, pre-opsonized in either NS or NS and IS, to proceed at a ratio of 100 bacteria per leukocyte. Both the ratio of bacteria to leukocytes (Leijh et al., 1980) and the type and degree of opsonization of the bacteria (Leijh et al., 1981) determine the number of bacteria ingested, which in turn affects the ability of the phagocytes to kill, i.e. in terms of the proportion of bacteria killed. This was most apparent in the present study, when the intracellular killing of B. vulgatus was examined (Tables 4.8 and 4.9). When pre-opsonized in 10% NS $2.0x10^5$ bacteria were phagocytosed by $5x10^6$ leukocytes and 84% of the bacteria were killed. However, when pre-opsonized in 10% NS and 10% IS $1.4x10^7$ B. vulgatus were ingested by the $5x10^6$ leukocytes and 54% were killed. Differences in the numbers of B. fragilis, B. vulgatus and E. coli phagocytosed in the 3 min period occurred (Table 4.8) and some of these can be attributed to the pre-opsonization treatment and, in the case of E. coli, to its ability to resist phagocytosis. The observation that significantly more B. fragilis, pre-opsonized in NS and IS, were phagocytosed in anaerobic conditions than in aerobic conditions may be either a statistical anomaly, or due to technical errors arising from time delays in carrying out manipulations in the anaerobic chamber. Furthermore, some phagocytic killing of bacteria occurs during the ingestion period (Leijh et al., 1981). This killing would be expected

to be reduced in anaerobic conditions (Table 4.9), resulting in a larger number of viable intracellular bacteria after 3 mins.

In the study of Leijh et al. (1980), when a strain of E. coli was phagocytosed at a ratio of 100 bacteria per granulocyte, 69.5% of the 5.9×10^7 bacteria ingested by the 5×10^6 granulocytes had been killed after 120 mins. When phagocytosed at a ratio of one E. coli per granulocyte, 96.7% of the 7.2×10^5 intracellular E. coli were killed. In their study, more S. aureus and E. coli were ingested and killed by human granulocytes at a ratio of 100 bacteria per granulocyte than were B. fragilis, B. vulgatus and E. coli by murine peritoneal leukocytes in the present study. This may be due to differences in the sources of leukocytes and serum (human cf. mouse respectively) and the strains of bacteria used. When confronted with large numbers of bacteria, the bactericidal mechanisms of phagocytic cells are less effective than when in the presence of low numbers of bacteria in terms of the proportion of bacteria killed (Clawson & Repine, 1976; Matheisz & Allen, 1979; Section 4.2.5a). Furthermore, the intracellular killing assays (Table 4.9) indicated that after 120 mins incubation in vitro, a population of viable bacteria existed within murine peritoneal leukocytes, with the potential for the bacteria to outlive their host cells, which were usually the short-lived neutrophils.

The strain of E. coli used in this study showed resistance to phagocytosis (Table 4.2) and intracellular killing (Table 4.9) by murine peritoneal leukocytes. The endotoxin of E. coli has been shown to reduce the bactericidal activity of human neutrophils when either pre-incubated with neutrophils or incubated simultaneously with neutrophils and other bacteria (Proctor, 1979). However, in the present study killed or live E. coli, a source of endotoxin, simultaneously

incubated with murine peritoneal leukocytes containing phagocytosed B. fragilis did not significantly affect the intracellular killing of B. fragilis (Tables 4.10 and 4.11). This may reflect differences between mouse and human phagocytic cells, but also B. fragilis showed some resistance to intracellular killing, which may have masked any effect of endotoxin.

4.3.3 Ultrastructural Observations on the Phagocytosed Bacteria

The ultrastructure of the phagocytosed bacteria (B. fragilis, B. vulgatus and E. coli) was similar in aerobic and anaerobic conditions despite more phagocytic killing having occurred in aerobic conditions (Section 4.2.4). In addition, the presence of IS, required for optimal phagocytic killing of B. fragilis (Fig.4.3a) and E. coli (Fig.4.3c), did not result in more damaged phagocytosed bacteria being observed. That the opsonins present and the oxygen status were not obviously reflected in the appearances of the bacteria was probably due to the time lag between loss of bacterial viability and ultrastructural damage to the bacteria (Mims, 1977).

Damaged B. fragilis were freguently observed within neutrophils in the absence of ongoing phagocytosis (i.e. intracellular killing assays, Section 4.2.5d). However, in a system of ongoing phagocytosis (i.e. phagocytic killing assays, Sections 4.2.5a and b), which more closely resembled conditions in the infected peritoneal cavity, B. fragilis withstood ultrastructural damage within murine peritoneal neutrophils for up to 120 mins in vitro. This was in contrast to B. vulgatus which was more frequently seen in a damaged state (Section 4.2.5b). The contrast between B. fragilis and B. vulgatus was apparent, despite ultrastructural observations being made in a system of

ongoing phagocytosis, i.e. a bacterium may have been morphologically intact because it had just been phagocytosed. That most B. vulgatus and a few B. fragilis were damaged was indicated mainly by the decreased electron density of the bacterial cytoplasm which sometimes gave it a vacuolated appearance. Since a change in the bacterial cytoplasm was the most frequently seen sign of damage, it was probably the first morphological alteration to occur in the phagosomes. Also seen were alterations to the morphology of the bacterial cell walls, which in places were distorted and disrupted. The end result of bacterial destruction was the appearance of cytoplasmic debris and remnants of cell walls in the phagosomes. This pattern of destruction has been reported for gram-negative (DeVoe et al., 1973a; Rozenberg-Arska et al., 1985; Young et al., 1985) and gram-positive (Goodman et al., 1956; Ayoub et al., 1969; DeVoe et al., 1973b) bacteria incubated with neutrophils in vitro.

The resistance of B. fragilis to ultrastructural damage in the present study was also evident when compared to B. intermedius, formerly B. melaninogenicus subsp. intermedius (Sundqvist et al., 1982). At a ratio of 50-100 bacteria per human neutrophil, B. intermedius was damaged after only 10-20 mins of phagocytosis. The resistance of B. fragilis to ultrastructural damage is further highlighted by other electron microscopy studies of phagocytosis, where damage to some intracellular gram-negative bacteria occurs within 10-20 mins of phagocytosis (Moore et al., 1978; Rozenberg-Arska et al., 1985; Young et al., 1985). Young et al. (1985) compared the in vitro phagocytosis and killing of two Brucella species by human neutrophils. Brucella melitensis, like B. fragilis, remained ultrastructurally intact during the 120 min assay period, whereas the Brucella abortus strain was damaged. Interestingly, B. melitensis induces the formation of micro-abscesses, whereas B. abortus causes granulomas to develop.

Both damaged and intact E. coli were seen in the phagosomes of neutrophils in vitro (Section 4.2.5c), albeit less frequently than the Bacteroides species, probably due to the poor phagocytosis of the E. coli strain used in this study (Table 4.2). Some neutrophils, although without visible intracellular E. coli in the plane of section under scrutiny, contained vacuoles which probably arose from phagocytosis. Bacterial debris accounted for some of the material within the vacuoles. However, the possibility that some of the vacuolation of the neutrophils was due to the toxicity of E. coli could not be discounted, given the potency of this species' endotoxin. The observation that some E. coli withstood ultrastructural damage is consistent with the study of Rozenberg-Arska et al. (1985). They showed structural changes occurred to only a few encapsulated E. coli within human neutrophils after 60 mins, whereas a non-encapsulated strain showed extensive damage after only 15 mins.

As the phagocytosis of bacteria proceeds, degranulation of the neutrophil granules in proximity to the developing phagosome begins (Zucker-Franklin & Hirsch, 1964). Bainton (1973) demonstrated that fusion of the secondary granules begins before fusion of the primary granules in rabbit neutrophils. However, immediately after the initiation of phagocytosis, the simultaneous degranulation of primary and secondary granules was observed within human neutrophils (Pryzwansky et al., 1979). In the present electron microscopy study, fusion of some primary granules with bacteria-containing phagosomes of murine peritoneal neutrophils was observed (Sections 4.2.5b and c). Peroxidase activity, the marker for primary granules, was seen in phagosomes containing either intact and damaged B. fragilis, B. vulgatus or E. coli. However, not all phagosomes in a plane of section contained peroxidase activity, and some peroxidase-negative phagosomes contained damaged bacteria. It is possible that, in some instances, phagosomes negative

for peroxidase in one plane of section may have been positive in another plane. Alternatively, primary granules may not have fused with the phagosomes at all. Furthermore, either some bacteria may have been recently phagocytosed, and there may have been insufficient time for degranulation to occur, or some bacteria may have been phagocytosed after the supply of primary granules was exhausted. There are approximately 33 primary granules in a mature neutrophil from murine bone marrow (Ogawa et al., 1983). Exhaustion of the supply of alkaline phosphatase-labelled secondary granules after the phagocytosis of many E. coli within just 10 mins was suggested to be the reason for the alkaline phosphatase-negative phagosomes observed in rabbit neutrophils (Bainton, 1973). Another possible explanation for the existence of peroxidase-negative phagosomes in murine peritoneal neutrophils observed in the present study is the inactivation of the enzyme within the phagosomes, especially during long, i.e. 2 hr, periods of incubation.

Secondary, i.e. peroxidase-negative, granules were also seen in the cytoplasm of some neutrophils which had phagocytosed bacteria, but in this study fusion of the secondary granules with phagosomes was not examined, due to the lack of a suitable method for labelling the granules for electron microscopy purposes (Section 3.2.4). However, demonstration of the fusion of some primary granules with phagosomes was significant, since they contain most of the microbicidal substances (Elsbach, 1980) and the secondary granules have been shown to contribute little to the killing of bacteria (Rest et al., 1978; Wang-Iverson et al., 1978). Despite fusion of some primary granules with 13-15% of B. fragilis-containing phagosomes (Table 4.7), the majority of the bacteria were still intact after 120 mins in vitro. The intact nature of B. fragilis could be due to the fusion of an insufficient number of primary granules with bacteria-containing phagosomes, or to the resistance of B. fragilis to

primary granule contents after degranulation has occurred. There may be some resistance to oxygen-dependent killing by neutrophils since some strains of B. fragilis synthesize a superoxide dismutase which has been linked to the oxygen tolerance of the organism (Tally et al., 1977; Gregory et al., 1978; Rolfe et al., 1978). However, a lack of extensive primary granule fusion with phagosomes would not expose B. fragilis to high concentrations of granule components which function in anaerobic conditions. Clinical isolates of B. fragilis were susceptible to oxygen-independent killing by granule extracts from human neutrophils (Pruul et al., 1983; Wetherall et al., 1984) whereas, the murine strain of B. fragilis used in the present study was resistant to killing by intact peritoneal leukocytes in anaerobic conditions (Table 4.4, Fig.4.4).

Extracellular serum is necessary for the intracellular killing of bacteria by neutrophils (Leijh et al., 1981) and in the present study, it was observed that upon phagocytosis of pre-opsonized B. fragilis in the presence of extracellular NS, which supported intracellular killing of B. fragilis (Table 4.9), peroxidase-positive and peroxidase-negative granules were rarely seen in the cytoplasm of neutrophils, indicating degranulation had occurred (Table 4.6). In contrast, when pre-opsonized B. fragilis were phagocytosed in the absence of NS, which did not support intracellular killing of B. fragilis, there were still many peroxidase-positive and peroxidase-negative granules present. This would suggest that the presence of extracellular NS, required for intracellular killing, influenced primary granule fusion with B. fragilis-containing phagosomes of neutrophils. In contrast, although more aerobic phagocytic killing of B. fragilis occurred in NS and IS than in NS alone (Fig.4.3a and Fig.4.4), the presence of IS did not increase the proportion of primary granules fusing with B. fragilis-containing phagosomes (Table 4.7). At a high ratio of bacteria to

leukocytes, the increased phagocytic killing in aerobic conditions of B. fragilis in the presence of NS and IS compared with NS alone (Fig.4.4) probably reflected the greater number of bacteria ingested in NS and IS, and therefore the larger number of phagosomes per neutrophil (Table 4.7), i.e. although the number of bacteria per phagosome and the proportion of peroxidase-positive phagosomes were similar to that in NS alone, it meant that overall more bacteria were exposed to neutrophil granule contents and therefore more were killed in NS and IS. Further investigations using biochemical methods and/or electron microscopy studies employing morphometric analysis to quantitatively determine the extent of degranulation and to establish the role of serum components in this process are warranted. Such methods have been used to show the phagocytosis of B. abortus strains by bovine neutrophils results in minimal degranulation of the primary and secondary granules (Riley & Robertson, 1984; Bertram et al., 1986).

The quantity and size of phagosomes containing B. fragilis varied from neutrophil to neutrophil. Some of this diversity may be attributed to the effect of the plane of section. However, the variation among the phagosomes was most apparent after 120 mins of phagocytosis in vitro. Individual neutrophils contained small and large phagosomes, with the large phagosomes holding many bacteria. The large phagosomes probably arose from the coalescence of small phagosomes over a period of time, and not from the phagocytosis of clumps of bacteria, because after 20 mins of phagocytosis in either NS or NS and IS, there was an average of only 1.6 bacteria per phagosome (Table 4.7). Further evidence for the fusing of phagosomes was the observation of mixtures of a few damaged bacteria, with intact bacteria within the one phagosome. It is not known whether the coalescence of the phagosomes is related to the fate of the intracellular bacteria. Similar observations were made in an in vitro electron microscopy

study of group B meningococci within human leukocytes (DeVoe et al., 1973a). The disruption of the cell membranes and phagosome membranes of necrotic neutrophils which appeared to free the intracellular bacteria was also seen in the present study after 120 mins of phagocytosis (Fig.4.14b). DeVoe et al. (1973a) also observed lysis of neutrophils which resulted in the extracellular release of bacteria-containing phagosomes. Clusters of gonococci, some of which were viable, have been seen enclosed in membranes of dead phagocytes in urethral pus (Novotny et al., 1975).

4.3.4 Conclusion

In an in vitro assay system of ongoing phagocytosis, akin to the situation in the murine peritoneal cavity, the fate of abscess-inducing bacteria was determined by several factors. The predominant cell involved in the phagocytosis of the bacteria was the neutrophil and both B. fragilis and B. vulgatus were readily ingested by neutrophils in NS in aerobic and anaerobic conditions. However, optimal phagocytic killing required oxygen, and in aerobic conditions B. vulgatus was killed in the absence of specific antibody, whereas B. fragilis was not killed. The proportion of peroxidase-positive phagosomes was similar in NS and NS plus IS, and therefore failure of phagosome-primary granule fusion was not the reason for the reduced killing of B. fragilis by neutrophils in NS alone. E. coli was not easily phagocytosed and specific antiserum was required for phagocytic killing. The potentiating agent, bran, reduced the killing of the bacteria by the peritoneal leukocytes through an effect on NS components, probably complement. Furthermore, at a high ratio of bacteria to leukocytes, a smaller proportion of B. fragilis were killed than at a low ratio of bacteria to leukocytes. Thus, after 120 mins in the presence of extracellular NS, a population of viable intracellular

bacteria still existed, despite the fusion of some primary granules with bacteria-containing phagosomes of the neutrophils. In addition, relatively few damaged B. fragilis were seen in phagosomes. It is suggested that an insufficient number of primary granules fused with B. fragilis-containing phagosomes. Moreover, by 120 mins, although the ultrastructure of some neutrophils appeared normal, other neutrophils had degenerated to the stage where cell membranes had ruptured, resulting in the release of bacteria from the phagosomes. At least some of the intact bacteria released were still viable.

In conclusion, whether bacteria survived an encounter with neutrophils in vitro was determined by properties of the bacteria and their numbers in relation to the concentration of leukocytes, the opsonins available for phagocytic killing and the effect of serum on post-phagocytic events within neutrophils which can be affected by bran and the oxygen available to the phagocytic cells.

CHAPTER 5

CONCLUDING DISCUSSION

The environment of an abscess is anaerobic (Hays & Mandell, 1974) but this does not alter the ability of neutrophils to phagocytose bacteria (Selvaraj & Sbarra, 1966; Mandell, 1974). In the present study, the phagocytosis of B. fragilis, B. vulgatus and E. coli was not affected by the availability of oxygen (Table 4.2). A ratio of 100 bacteria per peritoneal leukocyte was used in vitro because 1 hr after the IP inoculation of approximately $5x10^8$ bacteria, there was a range of $3-5x10^6$ peritoneal leukocytes per mouse (Table 3.3). At this ratio, B. fragilis and B. vulgatus were readily ingested in the presence of NS or NS and specific antisera in aerobic and anaerobic conditions (Table 4.2). The susceptibility to phagocytosis of this encapsulated murine strain of B. fragilis is consistent with some studies (Tofte et al., 1980; Ellis & Barrett, 1982; Bjornson et al., 1983) but not others (Simon et al., 1982). Capsular structures may determine the resistance of strains of Bacteroides species to phagocytosis rather than simply the presence of a capsule (Bjornson, 1984). This may explain why the encapsulated murine strains of B. fragilis and B. vulgatus (Fig.3.1) used in the present study were readily phagocytosed in NS in the absence of detectable antibody (Section 2.8). Of course, it is well established that the chemical composition of the capsule of many other bacterial species, e.g. Haemophilus influenzae type b (Roberts et al., 1981), influences their virulence. This may be the reason for the unique virulence properties attributed to B. fragilis capsules and not the capsules of other Bacteroides species (Onderdonk et al., 1977; Strohm et al., 1983; Kasper et al., 1984). However, other groups have found no correlation between the encapsulation of Bacteroides species and virulence (Babb & Cummins, 1978; Verweij-van Vught et al., 1986).

In this study of the phagocytic response of the murine peritoneal cavity to infection, the ability of neutrophils and macrophages to kill B. fragilis was much less than B. vulgatus.

This was evident both early and late in the infectious process. B. fragilis and B. vulgatus were equally susceptible to phagocytosis in vivo (Table 3.4) and viable B. fragilis or B. vulgatus, with E. coli, were found inside neutrophils and macrophages within the first 24 hrs of abscess development (Fig.3.25). However, 1 hr after the inoculation of the murine peritoneal cavity with abscess-inducing mixtures containing either B. fragilis or B. vulgatus, there were significantly more viable intracellular B. fragilis than B. vulgatus, and these differences were also significant 3.5 hrs later if bran had been included in the inoculum. Furthermore, B. fragilis and E. coli, phagocytosed in vivo within the first 24 hrs of infection, showed some resistance to killing by the peritoneal leukocytes when incubated with NS in vitro, whereas B. vulgatus appeared to be susceptible to killing (Table 3.6). In vivo, in neutrophils exposed to either B. fragilis (Fig.3.27a and b) or B. vulgatus (Fig.3.32), there was evidence of the fusion of some primary granules with bacteria-containing phagosomes, but more damaged intracellular B. vulgatus than B. fragilis were evident (Table 3.5). Ten weeks after the IP inoculation of mice with B. fragilis, E. coli and bran, IA abscesses containing viable bacteria at concentrations similar to those in the inoculum persisted (Table 3.1, Fig.3.2). In contrast, after just three weeks, the numbers of viable B. vulgatus in IA abscesses induced by the organism, in combination with E. coli and bran, were significantly reduced (Fig.3.3). In a study of a murine model of SC abscess formation, B. fragilis was also generally more virulent than B. vulgatus (Verweij-van Vught et al., 1986). These results are consistent with the clinical observation that B. fragilis is more pathogenic than B. vulgatus (Bartlett, 1981).

The resistance of intracellular B. fragilis to ultrastructural degradation by neutrophils was also evident in vitro, in the presence of ongoing phagocytosis at a high ratio of bacteria to peritoneal leukocytes. More intact B. fragilis than B. vulgatus

were observed within the phagosomes of neutrophils in both NS and NS plus IS (Section 4.2.5b). NS from C3H mice did not contain detectable antibody to B. fragilis and B. vulgatus (Section 2.8), and others have found only low titres of antibody to strains of Bacteroides species in murine NS (Foo & Lee, 1972; Ellis & Barrett, 1982). B. vulgatus was killed by phagocytes in NS alone (Fig.4.3b), whereas maximal phagocytic killing of B. fragilis and E. coli occurred at a ratio of one bacterium per ten peritoneal leukocytes in the presence of NS and specific antisera (Fig.4.3a and c). The addition of IS to NS resulted in slightly more phagocytic killing of B. fragilis at a ratio of 100 bacteria per leukocyte in aerobic conditions, and this was of statistical significance (Fig.4.4). Others have shown that the presence of complement and specific immunoglobulins facilitates the phagocytic killing of B. fragilis (Bjornson & Bjornson, 1978; Tofte et al., 1980; Ellis & Barrett, 1982). E. coli capsules or K antigens can also impose a requirement for specific immunoglobulins for the maximal phagocytic killing of strains of this organism in vivo (Ahlstedt, 1983) and in vitro (Guckian et al., 1978; Leist-Welsh & Bjornson, 1979; Horwitz & Silverstein, 1980). Thus, in the peritoneal cavity of a non-immune mouse, B. vulgatus would be more readily eradicated than would bacteria such as B. fragilis and E. coli, which require NS and specific immunoglobulins to be killed by phagocytes.

Ultrastructural evidence for the intracellular survival of B. fragilis was consistent with the lack of extensive phagocytic killing and therefore the survival of a greater proportion of B. fragilis, some of which were intracellular, at a high ratio of bacteria to peritoneal leukocytes (Fig.4.4, Table 4.5). Intracellular survival of some B. fragilis, after an initial period of phagocytosis at the high ratio, was also apparent when the numbers of viable intracellular bacteria were measured without further phagocytosis of bacteria (Table 4.9). These results are consistent with other studies which have demonstrated that the bactericidal mechanisms of phagocytes are

less effective, in terms of the proportion of bacteria killed when operating at high vs low ratios of bacteria to phagocytes (Clawson & Repine, 1976; Matheisz & Allen, 1979; Leijh et al., 1980). A limitation to the ability of neutrophils to kill all the bacteria they have phagocytosed was also evident in a study on the capacity of B. fragilis to inhibit the phagocytic killing of E. coli. Neutrophils which had already phagocytosed B. fragilis were unable to kill E. coli. Addition of another pyogenic organism, S. aureus, had the same effect as B. fragilis (Dijkmans et al., 1985).

The ability of some ingested bacteria to survive intracellularly is due to the inhibition of phagosome-lysosome fusion in macrophages (Armstrong & Hart, 1971; Oberti et al., 1981; Horwitz, 1983; Frehel et al., 1986; Frehel & Rastogi, 1987; Sibley et al., 1987) and this inhibition has been prevented by treatment of the bacteria with specific antibody (Horwitz, 1983; Frehel & Rastogi, 1987). Recently strains of B. abortus have been shown to induce minimal degranulation of the primary and secondary granules of bovine neutrophils (Riley & Robertson, 1984; Bertram et al., 1986). In this thesis the hypothesis that the presence of B. fragilis in neutrophil phagosomes inhibited the fusion of primary granules with phagosomes was tested in vitro. Primary granules contain most of the microbicidal substances of neutrophils (Rest et al., 1978; Wang-Iverson et al., 1978; Elsbach, 1980), whereas secondary granules contain the bacteriostatic agent, lactoferrin, and most of the cells' lysozyme, an enzyme considered important in degradation rather than killing of the bacteria (Root & Cohen, 1981). Inhibition of phagosome-primary granule fusion was not evident in either NS or NS and IS (Table 4.7) and therefore it was not the reason for the lack of phagocytic killing of B. fragilis in NS alone at a low (Fig.4.3a) or high (Fig.4.4) ratio of bacteria to peritoneal leukocytes. Although the addition of IS to NS, at a high ratio of bacteria to peritoneal leukocytes, did not alter the percentage of phagocytes with intracellular bacteria (Table

4.2), it did result in the phagocytosis of a greater number of bacteria (Table 4.7). Thus, in NS and IS, at the high ratio there were both more intracellular bacteria and more phagosomes per neutrophil, although the number of bacteria per phagosome and the proportion of peroxidase-positive phagosomes were similar to those in NS alone (Table 4.7). Consequently, overall more bacteria were exposed to granule contents in NS and IS, and more were killed by the peritoneal neutrophils than in NS alone (Fig.4.4).

A greater proportion of B. fragilis survived phagocytic killing at a high ratio of bacteria to peritoneal leukocytes than at a low ratio (Table 4.5). This result, plus the small proportion of peroxidase-positive phagosomes containing B. fragilis observed in either NS or NS plus IS, suggests that the fusion of an insufficient number of primary granules may influence the ability of the neutrophils to kill bacteria readily phagocytosed at a high ratio of bacteria to leukocytes. In the anaerobic environment of an abscess (Hays & Mandell, 1974), B. fragilis would be susceptible to phagocytosis by neutrophils (Table 4.2) but it would not be exposed to the complete oxygen-independent killing capacity of neutrophils if the ingestion of the organism resulted in the degranulation of a limited number of primary granules. This is consistent with the lack of significant killing of this virulent murine strain of B. fragilis in anaerobic conditions by intact leukocytes in vitro (Table 4.4; Fig.4.4) which contrasts with the susceptibility of clinical isolates to oxygen-independent killing by granule extracts from human neutrophils (Pruul et al., 1983; Wetherall et al., 1984). The ability of individual neutrophils to kill bacteria at a high ratio of bacteria to leukocytes may also be limited by the number of granules per neutrophil. Ogawa et al. (1983) demonstrated that mature neutrophils from the bone marrow of mice had a mean number of 33 primary granules and 148 secondary granules per neutrophil.

Intracellular killing has been studied in the absence of ongoing phagocytosis. Such studies have shown that extracellular serum is necessary for the stimulation of intracellular killing by human (Leijh et al., 1981) and murine (Hart et al., 1985) neutrophils. Heat-labile components of murine NS and immune serum facilitated the intracellular killing of P. mirabilis by murine neutrophils (Hart et al., 1987). The heat-labile components are probably part of the complement system because functional C3 receptors on neutrophils were shown to be necessary for the intracellular killing of P. mirabilis (Hart et al., 1986a). In the present study, it was observed that after the phagocytosis of pre-opsonized B. fragilis, in the presence of NS, few peroxidase-positive and peroxidase-negative granules were visible in the cytoplasm of neutrophils, indicating degranulation had occurred (Table 4.6). This correlated with intracellular killing of the majority of the bacteria (Table 4.9). In contrast, intracellular killing of pre-opsonized B. fragilis did not occur in the absence of NS (Section 4.2.5d) and there were many intact peroxidase-positive and peroxidase-negative granules within neutrophils. Intact granules were also observed in neutrophils which had phagocytosed pre-opsonized B. fragilis in NS which had been heated to destroy complement. This data suggests that the components of extracellular NS, probably complement, necessary for intracellular killing actually stimulated degranulation.

After 20 mins of in vitro phagocytosis in aerobic conditions, at a high ratio of bacteria to leukocytes in either NS or NS and IS, there was an average of 1.6 B. fragilis per phagosome (Table 4.7). However, after 120 mins, the coalescence of phagosomes containing B. fragilis appeared to have occurred. Large phagosomes contained several intact and damaged bacteria (Fig.4.13). The disintegration of membranes of necrotic neutrophils appeared to release these bacteria from the phagosomes (Fig.4.14b) and intracellular killing assays

indicated that some 20-40% of B. fragilis were viable at this time (Table 4.9). In vivo, released bacteria would be subject to phagocytosis by neutrophils attracted to the site of infection. However, the persistence of both abscesses (Table 3.1) and the bacteria they contain (Fig.3.2) for at least ten weeks, indicates the failure of the phagocytes to eradicate B. fragilis and E. coli from the murine peritoneal cavity. The presence of bacteria in extracellular (Figs.3.17a, 3.18b, 3.19 and 3.20) and intracellular (Figs.3.15 and 3.16b) sites within abscesses is also consistent with a cycle of phagocytosis, limited intracellular killing and then release of the bacteria. Since some bacteria are killed but the overall number of the bacteria in the abscesses remains fairly constant (Fig.3.2), there must also be bacterial replication. Limitations in the ability of phagocytes to kill the bacteria may also be due to a lack of opsonins within abscesses (Gordon et al., 1988). Similar observations have been made on the interaction of the pyogenic Neisseria meningitidis (DeVoe et al., 1973a) and Neisseria gonorrhoeae (Novotny et al., 1975) with neutrophils. Neutrophil-associated and extracellular gonococci are seen in urethral pus and Veale et al. (1977) also argue that due to the large number of neutrophils, it is unlikely the extracellular bacteria have never been phagocytosed.

Synergy between B. fragilis and E. coli has been demonstrated in animal models of IA sepsis (Table 1.1), including a murine model of IA abscess formation (Nulsen et al., 1983) which was used in this study. In the 24 hrs following the IP inoculation of mice with B. fragilis, E. coli and bran, a small number of viable E. coli persisted within the peritoneal leukocytes (Fig.3.25) and they were not killed upon incubation of the leukocytes with NS in vitro (Table 3.6). The number of viable intracellular E. coli was probably a reflection of both the small number of E. coli in the IP inoculum and the resistance of the organism to ingestion by neutrophils and macrophages, even in the presence of specific antiserum (Table 4.2). The persistence of E. coli

in intracellular and extracellular sites within the peritoneal cavity would provide a constant source of endotoxin. Bacteria were phagocytosed by macrophages in the peritoneal cavity (Table 3.4 and Fig.3.22). However, 24 hrs after the inoculation of abscess-inducing mixtures, necrotic macrophages were observed (Figs.3.29, 3.31, 3.34 and 3.36). The E. coli endotoxin may have affected the macrophages since endotoxin has a cytotoxic effect on human monocytes (Hammerström & Unsgaard, 1979) and murine peritoneal macrophages (Shands et al., 1974). Mononuclear phagocytes were detected in IA abscesses in C3H mice (Sections 3.2.3 and 3.2.5). However, these bands of cells did not expand as the abscesses aged as observed with abscesses in Balb/c mice (Nulsen et al., 1983). Only B. fragilis antigens were detected in the band of mononuclear phagocytes beneath the abscess granulation tissue (Fig.3.7). The presence of E. coli endotoxin in the abscess centre may have inhibited the spread of the mononuclear phagocytes. Necrotic neutrophils were not observed 24 hours after the IP inoculation of abscess-inducing mixtures, which does suggest that the mononuclear phagocytes were more sensitive than neutrophils to endotoxin in C3H mice.

A role for endotoxin was recently suggested in a mixed infection of the murine peritoneal cavity involving high doses of bacteria ($5x10^8$). The endotoxin of F. necrophorum, similar to the endotoxins of the Enterobacteriaceae, was thought to partially inhibit the host defence mechanisms, thus enabling the formation of chronic liver abscesses containing B. melaninogenicus (McCallum et al., 1983). At a concentration of 10 μg/ml or greater, E. coli endotoxin reduced the ability of human neutrophils to kill other bacteria (Proctor, 1979), but in the present study, killed or live E. coli did not significantly affect the intracellular killing of B. fragilis in vitro (Tables 4.10 and 4.11). However, a concentration of 10 μg of endotoxin per millilitre is equivalent to 10^9 bacteria per millilitre based on the calculations of Berry (1985), whereas the total number of viable E. coli in abscess-bearing mice never exceeded

10^7 (Fig.3.2).

In the murine model of IA abscess formation used in this study, bran was an essential component of the abscess-inducing mixture (Table 3.1, Fig.3.2 cf. Table 3.2, Fig.3.3). Bran, which is composed of polysaccharide-containing fibre (Saunders, 1978), was chosen as an alternative to the more complex mixture of autoclaved intestinal contents used as a potentiating agent in other models of IA sepsis (Onderdonk et al., 1976; Joiner et al., 1980b; Nulsen et al., 1983). Nulsen (1982) demonstrated reduced phagocytic killing of P. mirabilis, an organism opsonized by components of the alternative complement pathway (Finlay-Jones et al., 1984), in the presence of bran or autoclaved intestinal contents. This was postulated to be due to the competition between the organism and the potentiating agent for opsonization and phagocytosis. The current study showed that bran was phagocytosed in the murine peritoneal cavity (Fig.3.32) and in vitro (Fig.4.23). The presence of bran in vitro reduced the phagocytic killing of B. fragilis (Fig.4.24) and E. coli (Fig.4.25). Bran's effect on phagocytic killing involved some serum components, but not simply those required for opsonization since the bacteria were opsonized before addition to the assay mixtures. This suggests an effect on those serum components, probably complement, necessary for the stimulation of intracellular killing. The ingestion of both bran and bacteria may overwhelm the phagocytic capacity of the neutrophils. Fusion of granules with any phagosomes containing only bran would lead to a reduction in the number of primary granules available to fuse with those phagosomes containing bacteria.

The clumping of leukocytes observed in vitro (Fig.4.22) and in vivo (Fig.3.27a) in the presence of bran may also assist abscess development. Fewer peritoneal leukocytes were obtained by lavage 24 hrs after the IP inoculation of abscess-inducing mixtures containing bran (Table 3.3) which may have been due to

enhanced adherence of the leukocytes to the visceral surfaces. This effect of bran may be similar to the clumping factor of some S. aureus strains (Kapral et al., 1980). Thus, bran may potentiate abscess formation by enhancing the clumping of leukocytes leading to the trapping of bacteria within the peritoneal cavity, and also by reducing the intracellular killing of B. fragilis and E. coli within neutrophils.

Thus, B. fragilis persisted in IA abscesses in mice because it resisted killing by neutrophils after it was phagocytosed and contained within phagosomes. In the presence of large numbers of bacteria, the fusion of an insufficient number of primary granules with bacteria-containing phagosomes would contribute to the intracellular survival of some bacteria. A reduction in the supply of extracellular serum components, e.g. by bran, necessary for intracellular killing, possibly due to the promotion of phagosome-primary granule fusion, would further contribute to the survival of the phagocytosed bacteria. The subsequent release of these bacteria from necrotic neutrophils and the establishment of a cycle of phagocytosis, limited intracellular killing and release of bacteria with limited bacterial replication, would perpetuate the infection resulting in the development of chronic IA abscesses.

APPENDIX

MATERIALS

Bacto-Tryptone	Difco Laboratories Detroit, Michigan, USA
25% Glutaraldehyde	Merck Darmstadt, West Germany
Lead citrate	Serva Heidelberg, West Germany
Osmium tetroxide	Johnson Matthey Chemicals Ltd Royston, England
Paraformaldehyde	May & Baker Ltd Dagenham, England
Sodium acetate, anhydrous	Scientific Supplies Ltd Auckland, New Zealand
Sodium cacodylate	BDH Chemicals Ltd Poole, England
Sodium chloride	May & Baker Ltd Dagenham, England
Sodium diethyl barbiturate	Sigma St Louis, Missouri, USA
Sodium hydroxide	May & Baker Ltd Dagenham, England
Sucrose	BDH Chemicals Ltd Poole, England
Uranyl acetate	BDH Chemicals Ltd Poole, England

METHODS

Unless otherwise stated, the following methods were obtained from Dickson, M.R. (1978) Manual of the Biological Electron Microscopy Unit, University of New South Wales, Sydney, Australia.

A. Karnovsky's Fixative
(3% formaldehyde + 3% glutaraldehyde)

20% formaldehyde *	15 ml
25% glutaraldehyde	12 ml
1.0 M sodium cacodylate	10 ml

The constituents were made up to 95 ml with distilled water and the pH adjusted to 7.4 with 0.1 N HCl. Distilled water was then added to give a final volume of 100 ml.

* Preparation of 20% (w/v) formaldehyde solution :

paraformaldehyde	10 g
distilled water	40 ml
sodium hydroxide	2-4 drops

The suspension of paraformaldehyde was heated to 70-80°C to dissolve the solid. The solution was then cooled quickly.

B. Absorption of Non-Specific Fluorescence with Acetone-Dried Mouse Liver Powder
(Kawamura, 1977)

Preparation of acetone-dried mouse liver powder:

1. Livers were collected from adult mice after exsanguination.

2. The livers were weighed (x g), cut into small pieces and washed with distilled water several times.

3. The livers were homogenized in a Waring blender with an equal volume of saline, taking care not to generate heat.

4. The homogenate was poured into a beaker and eight volumes (8x ml) of acetone were added while stirring.

5. The precipitate was collected by centrifugation (955 g, 10 mins), resuspended in 4x ml of

saline and kept at 0-4°C overnight.

6. The precipitate was then suspended in x ml of saline, 8x ml of acetone was added and the precipitate collected by centrifugation. This step was repeated until the haemoglobin pigment was no longer seen in the supernatant.

7. After washing with saline, 4x ml of acetone was added to the final sediment. This was left to stand for a while, then stirred and the supernatant removed. The procedure was repeated and then the sediment was subjected to suction to remove the acetone as completely as possible.

8. A dry powder was obtained by leaving the material spread on filter paper in an incubator or desiccator. The yield of powder was approximately 5% by weight of the starting material.

The immune serum was absorbed with the acetone-dried mouse liver powder as follows :

1. 100 mg/ml of dry powder was added to the immune serum, mixed and left to stand for 1 hr at room temperature.

2. The mixture was centrifuged at 17700 - 31500 g for 20-30 mins.

3. 50 mg/ml of dry powder was added to the supernatant and the procedure was repeated.

4. The supernatant was collected taking care not to contaminate it with the sediment.

5. The treated immune serum was either used immediately or stored at -20°C.

C. <u>0.1 M Sodium Cacodylate Buffer, pH 7.4</u>

Stock 1.0 M buffer

214 g of sodium cacodylate was dissolved in 1000 ml of distilled water

Working 0.1 M buffer

20 ml of 1.0 M sodium cacodylate and 12.4 ml of 0.1 N HCl were made up to 200 ml with distilled water.

D. 2% Osmium Tetroxide in 0.1 M Sodium Cacodylate Buffer

1. A 5% (w/v) stock solution of osmium tetroxide was prepared two days in advance using distilled water and stored at 0-4°C.

2. The fixative was prepared by mixing 4 ml of 5% osmium tetroxide with 1 ml of 1.0 M sodium cacodylate and 5 ml of distilled water. The pH was adjusted to 7.4 with 0.1 N HCl.

E. 2% Agar in Tryptone Medium
(Hayat, 1972)

Distilled water	100 ml
Bacto-Tryptone	1.0 g
NaCl	0.5 g
Agar	2.0 g

The ingredients were dissolved by boiling. The mixture was dispensed in 1-2 ml aliquots and autoclaved (121°C, 15 mins).

F. Staining of Sections on Grids

Stains

2% uranyl acetate in 50% ethanol

This was prepared by mixing together equal volumes of 4% aqueous uranyl acetate and 100% ethanol and was kept in a foil-covered bottle.

Lead Citrate
(Venable & Coggeshall, 1965)

lead citrate	0.04 g
CO_2-free distilled water	10.0 ml
10 N sodium hydroxide	0.1 ml

The ingredients were dissolved by shaking in a stoppered bottle 24 hrs before required.

Staining

The grids were floated, sections facing downwards, on the 2% uranyl acetate alcoholic solution in the wells of a ceramic plate for 1 min. This was followed by thorough rinsing in 50% ethanol and one rinse in distilled water. The grids were washed in

running distilled water and then placed on drops of the lead citrate solution, on a piece of parafilm in a petri dish, for 1 min. The grids were rinsed three times in the wells of a ceramic plate and washed in running distilled water. The grids were left to dry on filter paper in a petri dish. If the specimens had been stained en bloc with uranyl acetate, the sections were stained with the lead citrate solution only, for 2 mins.

G. 1.5% Glutaraldehyde in 0.1 M Sodium Cacodylate Buffer, pH 7.4 with 1% Sucrose

25% glutaraldehyde	0.6 ml
1.0 M sodium cacodylate	1.0 ml
distilled water	7.0 ml
sucrose	0.1 g

After the sucrose had dissolved, the pH was adjusted to 7.4 with 0.1 N HCl, and the volume was made up to 10 ml.

H. 0.05 M Veronal Acetate Buffer, pH7.4
(A C Midwinter, personal communication)

sodium diethyl barbiturate	2.7 g
sodium acetate, anhydrous	1.33 g
0.1 N HCl to pH7.4	101 ml

The buffer was made up to 500 ml with distilled water. Sucrose was added to give a concentration of 7 g/100 ml if required.

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