STUDIES ON EXPERIMENTAL ANAEROBIC INFECTIONS OF THE MIDDLE EAR AND ON THE POLYMORPHONUCLEAR LEUKOCYTE FUNCTION UNDER ANAEROBIC CONDITIONS
ABSTRACT

Despite the clinical importance of anaerobic bacteria in otitis media and the uncertainty regarding the proper treatment of the anaerobic focal infection, few experimental studies focused upon the role of these microorganisms in otitis media have been published. In the present investigation a guinea-pig model for the induction of anaerobic monoinfections in the middle ear was described. Bacteroides fragilis and Propionibacterium acnes (4.0-10x10^7 colony forming units) injected via the tympanic membrane were capable of inducing clinical and histological otitis media with persistent sequele in the middle ear cavity. Bacteroides asacc-harlyticus, Peptostreptococcus micros and Peptostreptococcus anaerobius failed to induce otitis media. B. fragilis otitis was accompanied by increased serum IgG and IgM antibody titres against the challenge organism, whereas P. acnes and P. anaerobius did not induce a humoral immune response. The results suggested true virulence of B. fragilis in guinea-pig middle ear monoinfections.

Metronidazole was found to accelerate the elimination of B. fragilis from the middle ear. However, even high doses of metronidazole were not fully effective perhaps reflecting an incomplete anaerobiosis at the site of infection in some instances. At present, nitroimidazoles in chronic otitis media must be regarded as a possible alternative requiring further study, particularly with regard to the dosage.

In order to gain further knowledge of the interaction between polymorphonuclear leukocytes and bacteria under anaerobic conditions an in vitro model was established. It was shown that P. acnes was readily phagocytosed with the aid of C3 activated either via the classical or alternative pathway and that killing of P. acnes was inefficient during anaerobiosis. The results suggest that P. acnes is maintained in the pus in chronic otitis media because it survives phagocytosis.

Finally, the interaction between the most common pathogen in acute purulent otitis media, Streptococcus pneumoniae, and human polymorphonuclear leukocytes under anaerobic conditions was studied. Since purulent maxillary sinus effusion (and probably also purulent middle ear effusion), invariably has a pO_2 approaching zero, such studies are highly relevant with regard to the host defence in sinusitis and perhaps also in otitis media. S. pneumoniae was killed by the phagocytes under anaerobic conditions although at a slower rate than in air. Degradation of pneumococcal teichoic acid, DNA and RNA took place after phagocytosis under aerobic as well as anaerobic conditions, whereas degradation of unsaturated cell membrane lipids took place only under aerobic conditions. Furthermore, the pneumococcal autolytic system did not participate in the killing or the degradation of the bacteria.

Key words: Otitis media, anaerobic bacteria, experimental, nitroimidazoles, antibody, phagocytosis, anaerobiosis.
STUDIES ON EXPERIMENTAL ANAEROBIC INFECTIONS OF THE MIDDLE EAR AND ON THE POLYMORPHONUCLEAR LEUKOCYTE FUNCTION UNDER ANAEROBIC CONDITIONS

BY

MAGNUS THORE
TO THE MEMORY OF MY FATHER
Anaerobic bacteria are commonly present in the effusions of chronic otitis media as part of a polymicrobial infection, and such bacteria may rarely induce acute purulent otitis media. Severe complications secondary to acute or chronic otitis media are sometimes due to spreading of anaerobic bacteria from the middle ear, and may occur in the central nervous system (epidural or subdural abscess, meningitis or brain abscess etc) as well as in other parts of the body (septicaemia, pulmonary gangrene, purulent arthritis etc). The frequent occurrence of anaerobic bacteria in chronic otitis media has led to the concept of specific therapy in order to eliminate the anaerobic middle ear flora, and nitroimidazoles (i.e. metronidazole) may seem to be a reasonable choice of additive regimen. Despite the clinical importance of anaerobic bacteria in otitis media and the uncertainty regarding the proper treatment of the anaerobic focal infection, few experimental studies focused upon the role of these microorganisms in otitis media have been published. In the present investigation a guinea-pig model for the induction of anaerobic monoinfections in the middle ear was described. Bacteroides fragilis and Propionibacterium acnes (4.0-10^10 colony forming units) injected via the tympanic membrane were capable of inducing clinical and histological otitis media with persistent sequelae in the middle ear cavity. Bacteroides asaccharolyticus, Peptostreptococcus micros and Peptostreptococcus anaerobius failed to induce otitis media. B. fragilis otitis was accompanied by increased serum IgG and IgM antibody titres against the challenge organism, whereas P. acnes and P. anaerobius did not induce a humoral immune response. The results suggested true virulence of B. fragilis in guinea-pig middle ear monoinfections. Metronidazole was found to accelerate the elimination of B. fragilis from the middle ear. However, even high doses of metronidazole were not fully effective perhaps reflecting an incomplete anaerobiosis at the site of infection in some instances. At present, nitroimidazoles in chronic otitis media must be regarded as a possible alternative requiring further study, particularly with regard to the dosage.

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The present dissertation is based on the following papers which will be referred to in the text by their roman numerals.


VI Anaerobic phagocytosis, killing and degradation of \textit{Streptococcus pneumoniae} by human peripheral blood leukocytes. Submitted for publication. Magnus Thore, Sture Löfgren, Arne Tärnvik, Tor Monsen, Eva Selstam, Lars G Burman.

Illustration on cover: Chronic otitis media with a large epitympanal cholesteatoma. Drawing by the author.
ABBREVIATIONS

AOM = acute purulent otitis media
COM = chronic otitis media
SOM = serous otitis media
MOM = mucoid otitis media
CSOM = chronic secretory otitis media
MEE = middle ear effusion
MIC = minimum inhibitory concentration
IFL = immune fluorescence
IgG, IgM and IgA = immunoglobulin of classes G, M and A
C = serum complement
PMN = polymorphonuclear leukocyte (neutrophile granulocyte)
MDZ = metronidazole
CNS = central nervous system
BHI = brain heart infusion
CFU = colony forming units

DEFINITIONS

AOM = inflammation of the middle ear mucosa with a reddened tympanic membrane, seropurulent or purulent MEE, and pain.

COM = an inflammatory process in the middle ear accompanied by irreversible tissue damage and often by persistant perforation of the tympanic membrane, intermittent serous or purulent MEE and sometimes by cholesteatoma.

SOM = inflammation of the middle ear mucosa with a pale tympanic membrane, no pain. Clear or amber coloured MEE of low viscosity.

MOM = same as SOM except for presence of viscous MEE.

CSOM = longstanding (several months and even years) low viscous or viscous MEE behind an intact tympanic membrane, sometimes accompanied by atrophy of the tympanic membrane and tympanosclerosis.

Anaerobic bacteria = bacteria that requires a reduced oxygen tension for growth and fail to grow on the surface of solid media in 10% CO₂ in air (18% O₂).

Aerobic bacteria = bacteria able to grow in air only, or both in the presence and absence of air.
INTRODUCTION

MICROBIOLOGY OF THE MIDDLE EAR EFFUSIONS IN OTITIS MEDIA.

AOM. Streptococcus pneumoniae, Haemophilus influenzae and Branhamella catarrhalis are the predominating causative agents in AOM (Howie et al 1970, Kamme et al 1971, Schwartz et al 1977, Brook 1979). Also beta-haemolytic streptococci, Staphylococcus aureus and possibly S.epidermidis may induce AOM (Feigin et al 1973). Surprisingly little attention has been paid to the possible role of anaerobic bacteria in AOM, and most studies have not included anaerobic culturing despite that the MEE was culture negative in 20-30% of AOM cases.

While anaerobic bacteria probably are not a common cause of AOM, occasional reports suggest that such organisms are involved in AOM (see review by Finegold 1977), the first report dating back to 1901 (Rist, quoted by Finegold 1977). Cases of severe anaerobic AOM with complications were recently published (Moloy 1982, Krockmalska and Sydov 1982) and Brook (1979) isolated anaerobic bacteria (Peptococci, Peptostreptococci and Propionibacterium acnes predominated) from the purulent MEE of 27% of 186 children suffering from AOM. In 12.8% of the cases anaerobes were the only organisms isolated. However, in other studies attempts to isolate anaerobic bacteria from MEE aspirates have failed (Luotonen et al 1982).

COM. It has been known for a long time that anaerobic bacteria are commonly present in the discharge in COM as part of a polymicrobial infection, and anaerobic complications secondary to COM were described as early as in the nineteenth century (Rist, Thesis 1898, Guillemot, Thesis 1899, quoted by Finegold 1977).

frequently occurred. Especially in COM complicated by cholesteatoma anaerobes in pure culture were likely to be isolated.

Sugita and coworkers (1981) pointed out that anaerobic bacteria are most often isolated together with *S. epidermidis*, Corynebacteria and *Proteus mirabilis*, whereas *S. aureus* and *Pseudomonas* species predominated in ears negative for anaerobic bacteria. Swee­ney and coworkers (1982) found that the main bacterial species were present in the pus in counts which averaged $10^9$ organisms per ml. *Pseudomonas* species regularly showed higher counts of $10^{11}$ bacilli per ml.

**SOM, MOM (CSOM)** In 1958, Senturia and coworkers isolated aerob­ic bacteria from about 40% of the effusions in tubotympanitis. Since then several reports on isolates of staphylococci, *S. pneumonieae*, *H. influenzae* and *B. catarrhalis* in SOM, MOM (CSOM) have been published (Kokko 1974, Liu et al 1975, Giebink et al 1979, Riding et al 1978, Sundberg et al 1981). However, the role of bacteria (and certainly that of anaerobic bacteria) in SOM and MOM is still not established. Sipilä and coworkers (1981) found that in CSOM only few effusions yielded aerobic or anaerobic pathogenic bacteria, and Teele and coworkers (1980) failed to iso­late anaerobic bacteria from 51 MEE samples of CSOM. In sharp contrast, Fulghum and coworkers (1977) found several bacterial spe­cies in 10 cases of chronic secretory otitis media. Twentytwo % of the isolates were anaerobic and *P. acnes* dominated the findings. Purulent exacerbations of chronic secretory otitis media (behind an intact tympanic membrane) may be due to anaerobic bacteria (Brook 1979\(^a\), 1979\(^b\) and 1980).

**COMPLICATIONS SECONDARY TO OTITIS MEDIA WITH SPECIAL REFERENCE TO ANAEROBIC BACTERIA**

In the preantibiotic era CNS complications secondary to otitis media were more common than they are today. In 1935 Kafka reviewed 3225 patients with acute or chronic mastoiditis. Two­hundred and nine (6.4%) developed intracranial complications (meningitis 48%, lateral sinus thrombosis or subdural abscess 22.5%, brain abscess 16.5% and other complications 13%). The resulting mortality was 76.4%. Unfortunately, this report gives no information regarding
microbiological analyses of the material. In the early antibiotic era the death rate from acute or chronic ear disease was only 10% of the rate recorded in the preantibiotic era (Courville 1953). Today the overall mortality in intracranial complications of otitis media is about 10%, most of the complications being meningitis due to S. pneumoniae or H. influenzae (Gower and McGuirt 1983).

Severe complications secondary to AOM and COM are sometimes due to spreading of anaerobic bacteria from the middle ear and may occur in the CNS as well as in other parts of the body. Temporal lobe brain abscesses are in most cases (up to 70%) secondary to COM, and occur in particular when the ear disease is complicated by cholesteatoma. Such brain abscesses are caused mainly by anaerobic bacteria (Heineman and Braude 1963, Finegold 1977, Ingham et al. 1977, Cerny et al. 1981, Kramer et al. 1981). Less frequently they are secondary to AOM (Finegold 1977, Krockmalska and Sydov 1982). Among the anaerobic bacteria Bacteroides species (notably B. fragilis), anaerobic streptococci and Fusobacteria predominate, but also Clostridium ramosum and occasionally Propionibacteria may occur. Ingham and coworkers (1977) studied 9 cases of otogenic brain abscesses and a mixed flora of aerobic and obligate anaerobic bacteria was isolated from the pus in five patients and in the remaining four obligate anaerobes were the sole isolates. Bacteroides fragilis was present in all but one patient. Brain abscess secondary to middle ear and mastoid disease is usually the result of a direct extension of the infection by bone destruction or invasion via regional vessels and may not only be situated in the temporal lobe but also in the cerebellum or unusually in other parts of the brain. In an unpublished report from the southern part of Sweden about 20% of all brain abscesses recorded during the period 1947-1982 was secondary to otitis media. This material further showed that during the last 10 years 5/46 brain abscesses were secondary to otitis media, and occurred only in patients aged 40 years or more. Thus, the relative incidence of otogenic brain abscess has decreased and today a majority of brain abscesses are cryptogenic (C.-H. Nordström 1984, personal communication).

AOM and COM may also be complicated by other anaerobic or mixed infections such as subperiostal abscess, septic thrombosis of the lateral venous sinus and jugular vein, epidural or subdural
abscess or meningitis. These complications may occur singly or in combination, and also together with a brain abscess. Otitis media is the most common cause of anaerobic meningitis which develops as a direct extension of COM or secondary to a ruptured brain abscess. Fusobacteria and B. fragilis predominate the microbiological findings (Finegold 1977, Moloy 1982, Siegler et al 1982).

The diagnostic facilities and treatments currently available have greatly improved the outcome of intracranial complications secondary to otitis during the last decade, but when they occasionally occur they still constitute a considerable clinical problem (Gower and McGuirt 1983). Besides surgical drainage of brain abscesses (when possible), early diagnosis by computerized tomography and more effective antimicrobial drug regimens including nitroimidazoles (see below) have contributed to this favourable situation (Fig 1). The overall mortality in brain abscess in southern part of Sweden is currently about 5% (C.-H. Nordström 1984, personal communication).

Fig. 1. Computerized tomogram of the brain of a 26 years old female with chronic otitis media of the left ear complicated by a brain abscess of the left temporal lobe. Surgical intervention and total extirpation of the abscess resulted in complete recovery. The picture was kindly supplied by C.-H. Nordström, Department of neurosurgery, University of Lund, Lund, Sweden.
Other anaerobic complications secondary to otitis media may be osteomyelitis and Bezold abscess (Moloy 1982), septicaemia caused by Bacteroides species or Fusobacteria (Felner and Dowell 1971, Finegold 1977, Beeden and Willis 1980) and purulent arthritis (Finegold 1977). In tropical countries tetanus sometimes may origin from chronically discharging middle ears (Black and Atkins 1972). Secondary pulmonary gangrene was reported by Guillemot in his Thesis in 1899 (quoted by Finegold 1977).

ASPECTS ON ANTIMICROBIAL THERAPY WITH SPECIAL REFERENCE TO ANAEROBIC BACTERIA IN COM

The frequent occurrence of anaerobic bacteria in COM has led to the concept of specific therapy in order to eliminate the anaerobic middle ear flora (Jokipii and Jokipii 1981). Strains of these anaerobic species have been shown to develop resistance against tetracyclines, which in the past have been considered to be the drugs of choice in anaerobic infections, and which are widely used today in the treatment of COM. Most Bacteroides fragilis isolates as well as some other Bacteroides species and Fusobacteria produce betalactamases (Keush and O'conell 1966, C.-E. Nord, personal communication). Since betalactamase resistant penicillin derivatives are not as active as bezylpenicillin against sensitive anaerobes, and cephalosporines show varying or poor activity against B. fragilis (C.-E. Nord, personal communication) other drugs than betalactams must be considered. Nitroimidazoles are highly active against anaerobes with the exception of Propionibacterium acnes (Dornbusch et al 1979). The efficacy is comparable to that of clindamycin in anaerobic intraabdominal infections (Collier et al 1981). However, the in vitro effect against B. fragilis is cidal and greater than that of clindamycin (Selkon 1979, 1981). Occasional B. fragilis strains are resistant to clindamycin. Brook (1979b, 1980) found clindamycin to be of limited value in exacerbations of chronic secretory otitis media in children, though concentrations of the drug in MEE were not measured. In MEE the metronidazole concentration is about 70 % of that in serum (Jokipii and Jokipii 1981). Thus, nitroimidazoles would seem to be a reasonable choice as an adjunct to currently used antibiotic regimens. Since anaerobes, irrespective of their role in otitis media are actively involved in serious complications, their elimination is
desirable, In addition, this may contribute to successful management of the focal infection.

Nitroimidazoles. General considerations. Nitroimidazoles were originally recognized as antimicrobial agents with antiprotozoal activity, and were introduced into clinical medicine in 1960 for the treatment of *Trichomonas vaginalis* infections (Cosar and Julou 1959, Durel et al 1960). Davies and coworkers (1964) first noted that metronidazole was active against an anaerobic bacterium *Bacteroides necrophorus* (*Fusobacterium necrophorum*). Since then investigations have demonstrated the *in vitro* activity of MDZ, tinidazole and ornidazole against a variety of anaerobic bacteria (Dornbusch and Nord 1974, Goldstein et al 1978, Tally et al 1983). *Bacteroides fragilis*, *Bacteroides* species, *Fusobacterium* species and clostridia are uniformly susceptible whereas anaerobic grampositive cocci may show varying degrees of resistance. *Propionibacterium acnes* and aerobic bacteria are generally resistant to nitroimidazoles. Incomplete anaerobiosis in laboratory susceptibility testing can lead to erroneous results (false resistance in susceptible organisms, Milne et al 1978).

The mode of action of nitroimidazoles is not fully understood. The drug enters the eukaryotic (e.g. mammalian) as well as the prokaryotic (e.g. bacterial) cell by passive diffusion (Müller 1981). MDZ seems to act as electron acceptor from reduced ferredoxin and the nitrogroup of the drug becomes reduced (Edwards and Mathison, 1970). The reduced drug inhibits the DNA function, probably by attacking its thymine residues resulting in strand breakage (Edwards et al 1982). Other mechanisms (inhibition of DNA synthesis) may be involved in the killing of *B. fragilis* by MDZ (Sigeti et al 1983). No such activities have been observed in mammalian cells, although the possibility of a mutagenic effect of nitroimidazoles has been debated (Müller 1981).
Nitroimidazole derivatives are almost completely absorbed upon oral intake (Houghton et al 1979) and the serum half life of MDZ upon iv administration in humans is about 8-10 h whereas the corresponding value of tinidazole is about 10-15 h. These drugs bind to plasma proteins to only a limited extent (<20%, Sanvordeker et al 1975), and are distributed to virtually all tissues and body fluids in concentrations which do not differ markedly from corresponding serum levels (Andersson 1981). Nitroimidazoles also penetrate into cerebrospinal fluid and into the middle ear discharge in COM and are metabolised by side chain oxidation, hydroxylation and conjugation and mainly eliminated renally (Welling and Monro 1972).

HOST DEFENCE IN OTITIS MEDIA

Antibodies in AOM. S. pneumoniae, H. influenzae and B. catarrhalis induce a serum antibody response (Branefors-Helander et al 1973, Sloyer et al 1974, Branefors et al 1980, Leinonen et al 1981, Koskela et al 1982). Serum antibodies of IgG and IgM classes against the infecting organism have been shown to be of importance for the prevention of recurrent attacks of AOM (Karma et al 1980). Locally produced immunoglobulin (of IgG, IgM and IgA classes) may be of importance for the clearing of bacteria from the middle ear in AOM (Howie et al 1973, Sloyer et al 1974, 1976). Immune mechanisms in pneumococcal otitis media have been investigated in experimental models using chinchillas (see also General Discussion) and local as well as systemic immune factors have been demonstrated (Giebink et al 1976, 1977, 1979, Lewis et al 1980, Watanabe et al 1982a, 1982b).

Antibodies in COM. The presence of antibodies versus bacteria in human otitis media (COM) has been demonstrated by Hudac and coworkers (1983). These authors demonstrated low titres of Ig versus isolated anaerobic strains at the time of surgical intervention. So far, no experimental study regarding the humoral immune response to anaerobic bacteria in otitis media has been presented.
Polymorphonuclear leukocytes. General considerations. Whereas IgA primarily seems to interfere with bacterial adhesion to epithelial surfaces and thus acts in the first line of defence (Williams and Gibbons 1972), IgM and IgG are actively involved in the process of phagocytosis (reviewed by Horwitz 1982). Although different types of leukocytes may be active in otitis media (McGhee et al 1983), only the PMNs will be considered here, because they play the key role in the killing and elimination of invading microorganisms (Olsson and Venge 1980). The interaction between bacteria and PMNs may be divided into the following steps: chemotaxis, phagocytosis (attachment and ingestion), killing and degradation.

Chemotaxis. PMNs accumulate at the site of the infection due to a chemotactic process (Becker 1980). Various bacterial products (among which N-formyl-methionyl peptides are particularly potent, Schiffman et al 1975, 1978) or immune complexes may induce leukocyte chemotaxis. Chemotaxis may take place under aerobic as well as anaerobic conditions. Whereas immune complexes are chemotactic under both conditions, bacterial products are perhaps less chemotactic under anaerobic conditions (Kraal and Kenney 1975, Casciatio et al 1978). Also during activation of serum complement (see below), an additional chemotactic factor, the peptide C5a is formed (Ward and Newman 1969).

Phagocytosis of bacteria by PMN. The attachment of bacteria to the PMN membrane is facilitated by various serum proteins referred to as opsonins. Although IgG may serve as an opsonin, the most important opsonin is C3b, a fragment formed through cleavage of complement component C3 (Ehleuberger 1977) due to activation of the complement by bacterial surface structures (notably lipopolysaccharide) or by IgG or IgM-coated bacteria (Borsos 1971, Schultz 1980). Complement activation involving complement components C1-C4 is denoted "classical pathway" activation whereas initiation of complement activation at the C3 level is denoted "alternative pathway" activation.

PMNs have surface receptors for the Fc-portion of the IgG molecule and for C3b and may thus bind Ig and/or C3b coated bacteria (Ehleuberger 1977). After attachment has been established the PMN surface membrane invaginates and the attached bacteria are internalized.
in a vacuole (phagosome) (Hirsh 1962, Silverstein et al 1977). Phagocytosis takes place under aerobic as well as anaerobic conditions (Selvaraj and Sbarra 1966). Lysosomal granule contents are released onto the attached target or into the phagosome in order to kill and degrade the bacteria (Gabig 1981, Olsson et al 1981).

Killing of bacteria by PMN. In the presence of $O_2$, the interaction between bacteria and PMN leads to an increased metabolic activity with consumption of $O_2$ by the leukocyte ("respiratory burst", Sbarra and Karnovsky 1959). During this process reactive $O_2$ derived intermediates are generated e.g. $H_2O_2$ which in the presence of a catalizing enzyme (myeloperoxidase) may oxidate chloride ions, to form the highly bactericidal agent hypochlorous acid (Klebanoff 1968, Gabig 1981, Sips and Hamers 1981). Although many microorganisms are not efficiently killed by PMNs unless $O_2$ is present (Selvaraj and Sbarra 1966, Mandell 1974), PMNs seem to have also $O_2$-independent killing mechanisms active against e.g. E. coli and certain streptococcal species (Mandell 1974, Odeberg and Olsson 1975, Arnold et al 1980, Olsson et al 1981, Weiss et al 1982, Elsbach and Weiss 1981 and 1983). Lysosomal cationic proteins, lactoferrin or lysozyme are examples of bactericidal proteins which probably do not require $O_2$ for their activity. Acidification of the phagosome milieu may in itself be cidal i.e. to pneumococci (Lord and Nye 1919, Jacques and Bainton 1978). Some bacteria, not involved in otitis media (i.e. Legionella pneumophila and Francisella tularensis) are however notoriously resistant to intracellular killing by PMN (Horwitz and Silverstein 1981, Löfgren et al 1984).

Degradation of bacteria by PMN. The killed bacteria are finally degraded by the action of PMN enzymes with hydrolase activity (such as proteases, nucleases, phospholipases) and possibly also by bacterial autolytic enzymes (Elsbach et al 1973, Elsbach 1980, Ginsburg and Lahaw 1983). The bacteria may be incompletely degraded, and partially digested microbial remnants have been suggested to be important antigens sustaining the host's immune response (Elsbach 1980). To my knowledge, degradation of phagocytosed microorganisms has not been studied under anaerobic conditions despite the fact that purulent infections of the upper respiratory tract may create
an anaerobic milieu (see below and General discussion).

**PMNs in otitis media.** Purulent middle ear effusion is chemotactic to PMN (Bernstein and Byung 1981) possibly due to C5a formed during complement activation (Bernstein et al 1978). However, in otitis media PMN may not react normally to chemotactic stimuli and their capacity to kill pathogens may be depressed (Giebink et al 1980). Phagocytosis of e.g. pneumococci is dependent on antibody and complement (Johnston et al 1969, Smith and Wood 1969, Winkelstein et al 1973, Giebink et al 1977) but abnormal activation of complement has been observed in otitis media (Prellner and Nilsson 1982) which may lead to impairment of opsonization and of the subsequent phagocytosis. During PMN chemotaxis and phagocytosis proteolytic enzymes such as granulocyte elastase and chymotrypsine-like cationic proteins are released by the PMNs (Wright 1982). Such extracellular enzymes may be responsible for part of the tissue damage seen in otitis media by their action on collagen (Carlsson et al 1982) and they may also split e.g. immunoglobulines present in the effusions in COM (Kastenbauer et al 1975). Proteolytic enzymes (rather than the invading bacteria) are probably responsible for the tissue destruction seen also in sinusitis (Engquist et al, 1983). Furthermore, the reduced O2 tension in maxillary sinusitis (Carenfelt and Lundberg 1977, 1978) is most likely typical also of AOM and COM (see further General discussion). Although the capacity of PMNs to kill certain microorganisms may be impaired under anaerobic conditions (see above), phagocytosis and killing of P. acnes and S. pneumoniae have not earlier been studied under anaerobic conditions.
AIMS OF THE PRESENT INVESTIGATION

Because of the many unanswered questions regarding the roles of anaerobic bacteria in otitis media this study was initiated with the following aim:

To establish an animal model for induction of otitis media by different species of anaerobic bacteria allowing analysis of
the capacity of anaerobic bacteria to induce otitis media
the efficacy of MDZ in experimental anaerobic otitis media
the humoral immune response in experimental otitis media.

In order to gain further knowledge of the interaction between PMN and bacteria under anaerobic conditions an in vitro model was established with the aim to study

the role of serum complement in phagocytosis of P. acnes
the role of oxygen in granulocytic killing of P. acnes
the influence of anaerobiosis on phagocytosis and killing of encapsulated and unencapsulated strains of S. pneumoniae.
the influence of anaerobiosis on macromolecular degradation of encapsulated pneumococci
the role of the pneumococcal autolytic system in granulocytic killing of encapsulated S. pneumoniae.
MATERIALS AND METHODS

THE ANIMAL MODEL

Bacteria, growth conditions and preparation of bacterial suspensions (I, II, III and IV). Encapsulated type 23 S. pneumoniae were subject to serial subcultivation on blood agar plates (Oxoid, Hants, England) supplemented with increasing concentrations of gentamicin (1-64 mg per l). The gentamicin resistant mutant thus obtained was passed in a mice peritonitis model to maintain its virulence and was subsequently used for induction of a standard pneumococcal otitis media in guinea-pigs (see below).

Propionibacterium acnes NCTC 737, Bacteroides asaccharolyticus (formerly B. melaninogenicus subspecies asaccharolyticus), Peptostreptococcus micros (supplied by G. Sundqvist, University of Umeå, Umeå, Sweden), Bacteroides fragilis NCTC 9343 and Peptostreptococcus anaerobius ATCC 27337 were used as challenge organisms for the induction of anaerobic otitis media. The pneumococci were grown overnight at 37°C in sealed glass tubes with Trypticase yeast broth (Holm and Falsen 1967) whereas the anaerobic bacteria were grown on blood agar; in some series these plates were supplemented with gentamicin (100 mg per l) in order to avoid contamination (III). Culturing of anaerobic bacteria was performed under anaerobic conditions for 24-48 h in a 37°C anaerobic glove box (atmosphere of 10% H₂, 5% CO₂ in N₂) or in anaerobic jars (Gas Pak system, BBL Cockeysville, Md). None of the anaerobic strains was inhibited by 32 mg gentamicin per l. Immediately before a challenge experiment the bacteria were suspended in pH 7.5 HEPES-buffer (130 mM KCl, 2mM MgSO₄ and 50 mM N-2-hydroxyethylpiperazine-N-2 ethansulfonic acid, HEPES, Sigma Chemical Co., St. Louis) in deionized water at the desired concentration (see Results). Whether anaerobic or aerobic buffer was used did not influence on the 60 min viability of any of the microorganisms studied.

Induction of otitis media in guinea-pigs (I, II, III and IV). A total of 263 albino guinea-pigs (Nybergs Gård, Märsta, Sweden or HB Sahlins försöksdjursfarm, Malmö, Sweden, weight 250-450g) were used in the challenge experiments with buffer (n=27 animals, I)
S. pneumoniae (n=22 I) P. acnes (n=59 I, II and IV),
P. fragilis (n=106 II, III and IV) and with P. micros,
B. asaccharolyticus and P. anaerobius (n=49 animals I, II and IV). The animals were anesthetized by an intraperitoneal (ip) injection of 30 mg of pentobarbital/kg body weight and both ears were examined to exclude ongoing infections. The right external ear including the external ear canal was disinfected using 10% H₂O₂ for 30 sec followed by 3% I₂ in 70% ethanol for 30 sec. The I₂ was inactivated by sterile 10% sodium thiosulphate and a final wash with sterile water was performed. All animals included in the study had culture negative external ears after disinfection.

The bacterial suspension (0.1-0.2 ml) was injected through the tympanic membrane into the right middle ear (0.4x20 mm needle), leaving the left ear as an inoculated control. In order to reduce later contamination the external ear canal was sealed with a plug consisting of paraffin and petroleum jelly. In all bacterial challenge experiments a daily ip injection of 4.5 mg of gentamicin (Schering Corp. Kenilworth, N.J.) in 1 ml of sterile water was used as prophylaxis against aerobic superinfections (See I). The challenged animals were kept in separate, ventilated cages.

Sacrifice of guinea-pigs and bacteriologic sampling of ears (I, II, III and IV). At various intervals after bacterial challenge (2-35 days), the animals were killed using a lethal dose of pentobarbital. The external ears were removed and the temporal bones were surgically separated from the skull. The middle ears were entered through an inferior-posterior hole that was made using a pair of sterile bone tongs. After gross examination of the middle ears, any effusion present was aspirated and immediately diluted into 5 ml of thioglycolate broth (I) or phosphate-buffered saline (PBS, 12.6 mM KH₂PO₄, 54.0 mM Na₂HPO₄, 85 mM NaCl; Ph 7.4, papers II, III and IV). The middle ears were then washed with 0.1-0.2 ml of the fluid using the same syringe, and the wash fluid was added to the specimen. After appropriate dilutions in PBS the samples were spread over blood agar plates (in III the plates were supplemented with gentamicin 100 mg/l) and immediately incubated anaerobically (glove box or Gas Pak) and aerobically for 48 h at 37°C. The colonies obtained were counted and identified by morphology. For further identification of anaerobic bacteria Bacto
Corynebacterium acnes 554 antiserum (Difco, Detroit Mn), the API system for anaerobic bacteria (Montalieu-Vercieu, France) and the VPI manual (Virginia Polytechnic Institute, Va) were used. Aerobic bacteria were identified according to standard laboratory techniques.

Histologic examination of ears (I and II). On average every third infected middle ear was subjected to histological analysis. The excised temporal bones were fixed in 10% neutral formaldehyde and decalcified in New Decalk^R (Histo-Lab, Bethlehem Trading, Gothenburg) for 4-5 h. After dehydration the material was embedded in paraffin and sectioned. Thin sections were stained with Brown-Brenn stain (Sheehan and Hrapchak 1973, I) but unfortunately this method did not give reproducible results and hematoxylin and eosin was therefore used in II before light microscopy.

Metronidazole. Metronidazole (MDZ) was supplied by AB Leo Rhodia, Helsingborg, Sweden, as dry powder.

Assay of MDZ in guinea-pig serum (III). A Clostridium sporogenes strain was used in a microbiological assay based on diffusion in agar. Approximately $10^3$ cfu were seeded on BHI agar (Difco Laboratories Inc., Detroit, Mich.). Paper discs were used as diffusion centres for the 20 ul aliquots of serum assayed. Plates were read after 24 h incubation at 37°C under anaerobic conditions (Gas Pak). The detection limit of this bio-assay was 0.4 mg MDZ per l.

Serum antibody assay (IV). The indirect immunofluorescence testing (a modification of the technique described by Schwan and coworkers 1982) was used in order to detect antibodies of IgG, M and A classes in paired sera from 42 animals challenged with B. fragilis (n=23) P. acnes (n=12) and P. anaerobius (n=7) randomly selected among a total of 94 guinea-pigs (II). About $10^8$ formaldehyde killed bacteria were used as antigens suspended in 10 μl PBS smeared on glass slides (Novakemi AB, Stockholm). Appropriate dilutions of serum samples were prepared, and 10 ul was allowed to react with the antigen in a moist chamber (30 min, 37°C). After washing, FITC-labeled antisera (Nordic Immunological labs, B.V. Tillburg, The Netherlands) were added and the glass slides
were incubated in a moist chamber (20 min, 37°C). Phosphate buffered glycerol (Ph 7.2) was used as mounting solution and the reactions were studied in a Dialux 20, Leitz fluorescence microscope. All reactions were repeatedly judged at separate occasions by one individual unaware of the origin of the serum samples. The specificity of the antibody reaction was tested by adsorption with homologous bacterial antigen and preinoculation guinea-pig sera were used as negative controls.

INTERACTION BETWEEN PMNS AND BACTERIA

Bacteria (V and VI). The *P. acnes* strains studied were NCTC 737 and a clinical isolate designated MC-7. The five encapsulated *S. pneumoniae* strains used (of serotypes 2, 9N, 14, 21 and 23F) were clinical isolates, whereas the unencapsulated type 2 *S. pneumoniae* strain studied was ATCC 27336. For control purposes (see below) *Streptococcus faecalis* strain C3 and *Staphylococcus aureus* phage type 52/80 I were used.

Culture conditions (V and VI). *P. acnes* was cultivated on blood agar (OXOID) under anaerobic conditions (Gas Pak, paper V). Encapsulated pneumococci were cultivated in BHI broth (Difco). The unencapsulated type 2 pneumococcal strain was cultivated on blood agar. The control bacteria (*S. faecalis* and *S. aureus*) were cultivated on blood agar (V) or in BHI broth (VI). For the induction of autolysis resistant type 14 *S. pneumoniae* a defined medium (Tomasz 1964, 1968, paper VI) supplemented with ethanolamine (40 mg per l) instead of choline (4 mg per l) was used.

Radiolabelling of *S. pneumoniae* serotype 14 (VI). \( ^{14}C \) arachidonic acid (0.3 uCi per umole, Amersham International Ltd, Amersham, UK) was used for labelling of unsaturated cytoplasmatic membrane lipids. \( ^{14}C \) methyl choline chloride (58 uCi per umole, Amersham) or \( ^{14}C \) ethanol-01-2 amine hydrochloride (60 uCi per umole, Amersham) for teichoic acids and \( ^{14}C \) uracil (50uCi per umole, Amersham) for nucleic acids.

Sera (V and VI). A serum pool using blood from 4 healthy donors was prepared, stored in aliquots at -70°C (pooled normal human serum, PNHS). Serum was also prepared from blood of an adult hypo-
gammaglobulinemic individual, collected immediately before substitution therapy (HGS, paper V). Serum was further prepared from an individual vaccinated with Pneumovax^R (Merck, Sharp & Dohme, International, Rahway, N.J.) 4 weeks earlier. The antipneumococcal IgG, M and A titres of this serum were determined by M. Koskela, University of Uleåborg, Uleåborg, Finland. The IgG titre versus type 2 pneumococcal capsular polysaccharide after vaccination was 4-fold those of the corresponding titres in PNHS (VI).

Depletion of serum factors (V). Affinity chromatography with cyanogen bromide activated Sepharose 4B (Pharmacia AB, Uppsala, Sweden) was used for depletion of IgG and IgM from HGS and for the elimination of complement components C3, C4 and factor B from PNHS. Rabbit anti-human IgG antoglobulin or rabbit antihuman IgM antoglobulin (DAKO-Immunoglobulin A/S, Copenhagen, Denmark) coupled to Sepharose 4B was used for depletion of IgG and IgM. Goat antihuman C3 antiserum (Meloy Laboratories, Springfield, Va), rabbit anti-human C4 antiserum and rabbit anti-human factor B antiserum (DAKO-Immunoglobulin A/S) coupled to Sepharose 4B was used for depletion of C3, C4 or factor B.

Assay of serum factors (V). IgG of HGS and IgG-depleted HGS was quantitated using the enzyme-linked immunosorbent assay performed according to Carlsson and Lindberg (1978). IgA and IgM levels of the corresponding sera were determined using LC-partigen plates (Behringwerke AG, Hamburg, West Germany). IgG of other serum samples, C1, C3, C4 and factor B were quantitated by immunodiffusion using partigen plates (Behringwerke AG).

Assay of complement activation (V). The activation of complement by the alternative and classical pathways was determined using a technique based on hemolysis in gel of rabbit erythrocytes (Fong et al 1978) and antibodycoated sheep erythrocytes (Lange 1960) respectively.

Preparation of human peripheral blood leukocytes (V and VI). Heparinized venous blood from healthy volunteers was subjected to dextransedimentation (Skoog and Beck 1956). Erythrocytes were lysed using 0.87% ammonium chloride. The leukocytes were suspended in buffer at a density of 5x10^7 PMNs per ml (V
and VI). Further purification of PMNs from venous blood was obtained by the Hypaque-Ficoll technique (Ferrante and Thong 1978, paper VI). However, this preparation did not differ from the dextran sedimented leukocytic preparation in phagocytic or degradative capacity. Therefore dextran sedimented leukocytes were used in papers V and VI.

Assay of phagocytosis and killing (V and VI). Mixtures of leukocytes, serum and bacteria were used for studies of phagocytosis and killing of the bacteria according to Cohn and Morse (1959).

Assay of macromolecular degradation (VI). These studies were exclusively performed using radiolabelled (see above) type 14 pneumococci. For assay of degradation of unsaturated cytoplasmatic membrane lipids, lipid extraction according to Folch and coworkers (1957) was performed before the liquid scintillation counting of lipid label.

Label in teichoic and nucleic acids, was measured by precipitation by trichloroacetic acid (TCA) before collection of precipitates on Whatman GF/C glass microfibre filters (Whatman, Maidstone, UK) and subsequent scintillation counting.

Anaerobic technique and functional controls (V and VI). For anaerobic experiments an anaerobic glove box with an atmosphere of 10% H₂, 5% CO₂ in N₂ was used.

The redox potential of the anaerobic experimental cell mixtures was monitored with a freshly sanded platinum electrode using a H₂ electrode (electrodes P101 and K410, respectively, Radiometer, Copenhagen) as reference. The redox potential was -299±4 mV when assayed immediately before as well after 60 min of incubation of the anaerobic cell mixture (VI).
RESULTS

THE ANIMAL MODEL

Sensitivity of the guinea-pig middle ear to HEPES-buffer (I).
Upon injection of sterile HEPES-buffer into the right middle ear of 12 guinea-pigs, inflammation developed in five ears, of which beta-haemolytic streptococci group C were isolated from two. Another 15 animals were given daily ip gentamicin injections during the entire course of the buffer inoculation experiment. Otitis media did not occur under these conditions, and no bacteria were isolated from the ears. Gentamicin prophylaxis lowered the risk of otitis media associated with middle ear injections of buffer (p<0.05) and was therefore used in all of the experiments reported below.

Inoculation of low doses of anaerobic bacteria (I and II). In preliminary experiments 10^5 cfu was used as challenge dose of P. acnes, B. fragilis, and P. anaerobius. At this dose only P. acnes was recovered at sacrifice on day 5 after challenge but the counts were low.

Inoculation of high doses of anaerobic bacteria (I and II). Challenge doses of 4-8x10^7 cfu/ear were used in experiments using P. acnes, B. fragilis and P. anaerobius (II). On day 3, 5 and 10 after challenge P. acnes was isolated as single species from 15 of 21 ears studied, and from 3 additional ears together with aerobic bacteria (II). No evidence for multiplication of these anaerobic bacteria in the middle ear was found, and at day 12 and 35 P. acnes was never recovered (Fig 2).

On day 3, 5, 10 and 12 B. fragilis was isolated as single species from 20/28 ears studied (II) and occasionally B. fragilis was cultivable at still later stages. Thus, on day 18 and 21 pure cultures of B. fragilis were obtained from 7/12 ears sampled. In addition, B. fragilis together with staphylococci and enteric bacilli was cultured from the middle ear of another animal. The arithmetic mean values of the middle ear bacterial counts on different days after inoculation (Fig 2), indicated that B. fragilis, declined during the first week and multiplied in the middle ear.
during the second week. \textit{B. fragilis} was then gradually eliminated, and the organism was not recovered on day 35 after challenge (n = 4).

\textit{P. anaerobius} (II) was rapidly eliminated during the 5 days following challenge and was never found during the second week (Fig 2).

![Graph](image)

\textbf{Fig. 2.} Recovery of \textit{Bacteroides fragilis} (●) \textit{Propionibacterium acnes} (□ and ■) and \textit{Peptostreptococcus anaerobius} (▲) from the right middle ears of guinea-pigs on various days after inoculation. On day 0, the bacteria suspended in buffer were injected through the tympanic membrane. Bacterial counts less than 50 cfu per ear were not detected under the assay conditions employed, and such ears were recorded as culture negative (below dashed line). The symbols denote arithmetic mean values.

Even higher doses ($10^8$ cfu/ear) were applied in challenge experiments using \textit{P. acnes}, \textit{B. asaccharolyticus} and \textit{P. micros} (I). \textit{P. acnes} was recovered from middle ear specimens from 10/16 ears on day 2, 5 and 10 after challenge, in 3 of these ears together with other bacteria.

\textit{B. asaccharolyticus} and \textit{P. micros}, separately or in combination, were eliminated from the middle ears within 2 days.

\textbf{Throat flora organisms and secondary invasion (I and II).} Middle ear invasion by aerobic bacteria sometimes occurred upon challenge with anaerobic bacteria. In one series (I) about 30% of the ears
were secondarily invaded mainly by group C streptococci. Such organisms were often recovered from the throat flora of the animals before challenge (51%) and at sacrifice (31%) whereas streptococci were rarely found in external ears before disinfection. This invasion of streptococci despite antibiotic prophylaxis apparently originated from the throat via the eustachian tube and was possibly due to the relative tolerance to gentamicin of these bacteria (MIC of gentamicin = 8 mg/l). In another series (II) secondary invasion occurred in less than 10%, and betahaemolytic streptococci was isolated from only 1/94 challenged ears and never from the nasopharynx.

Gross examination of middle ears (I, II, III and IV). After sacrifice the opened middle ears were inspected and scored as 0 (non-inflamed), + (inflamed with reddish mucosa, but no MEE), ++ (varying amount of apparently purulent or non-purulent MEE) or +++ (vigorously inflamed i.e. filled with apparently purulent or non-purulent MEE). Since all of the effusions was used for bacteriological examination no systematic microscopic investigation of the MEE was performed regarding their cellular composition. However, preliminary results of microscopy showed that apparently purulent MEE was rich in PMNs whereas clear to amber MEEs contained few inflammatory cells.

Vigorous inflammation always developed upon challenge with $10^7$ cfu of type 23 pneumococci (I). Also *P. acnes* induced a vigorous inflammation after challenge with about $10^8$ cfu in preliminary experiments (I), but using this challenge dose a more heterogenous response to *P. acnes* regarding the amount and character of the MEE was found in a subsequent series of 16 animals. Lower doses of *P. acnes* were also used (II) and vigorous inflammation never occurred in this series of 28 animals.

*B. asaccharolyticus* and *P. micros* in pure cultures and in combination failed to induce MEE.

The inflammatory response to *B. fragilis* challenge (II) was pronounced. On day 3 purulent effusion was noted in 5/6 ears studied. On day 5, 4/7 ears studied contained small amounts of purulent effusion and 3 ears were inflamed but dry and on day 10 and 12, 10/15 ears studied contained varying amounts of purulent effusion.
The inflammatory reaction was usually less pronounced on day 18 and 21. On day 35 a small amount of effusion was noted in 1/4 ears. _P. anaerobius_ (II) failed to induce more than at best a weak inflammation in a minority of the ears challenged.

**Histology (I and II).** The histological picture in pneumococcal otitis media (I) was dominated by subepithelial oedema, accumulation of PMNs, and dilated blood vessels. However, no bacteria were ever seen within the subepithelial space. The histology of ears with a vigorous _P. acnes_ infection (challenge dose $10^8$ cfu/ear I) closely resembled the tissue response in pneumococcal otitis media. Mucosal swelling was more pronounced at day 2 than later and on day 5 early signs of new bone formation appeared. In contrast to pneumococcal ear infection bacteria were seen in the subepithelial space on days 2 - 5 during _P. acnes_ otitis. With lower challenge doses of _P. acnes_ (II) histological evidence of inflammation was less pronounced, but slight mucoperiostal thickening with new bone formation was noted in 1/4 ears 35 days after challenge.

During the early phase of _B. fragilis_ infection the histological picture was dominated by subepithelial oedema and infiltration by inflammatory cells, mainly PMNs (Fig 3, 4). Profuse new bone formation with extensive growth of periosteum was evident on day 10 - 12 in culture positive ears and was still present on day 35. The new bone never obliterated the middle ear cavity, but was often recognized as an increased difficulty in surgical penetration into the cavity.

Minimum or no histological signs of otitis were noted with _B. asaccharolyticus_ or with the two species of _Peptostreptococci_ studied (I and II).

While labyrinthitis occasionally occurred following pneumococcal challenge, no such complications were noted with any of the anaerobes used, and the animals never showed behavioural abnormalities upon challenge with anaerobic bacteria.
Fig. 3. Light micrograph of the sectioned middle ear of a guinea-pig challenged with Bacteroides fragilis (NCTC 9343) 3 days earlier. Note subepithelial oedema with infiltration of inflammatory cells. Hematoxylin and eosin.

Fig. 4. Light micrograph of the sectioned middle ear of a guinea-pig challenged with Bacteroides fragilis (NCTC 9343) 10 days earlier. Remaining pus in the middle ear cavity. Hematoxylin and eosin.

Serum kinetics of metronidazole (MDZ) in guinea-pigs (III). A single ip dose of 6 mg MDZ resulted in a peak serum level of
6 ± 1.4 mg active drug per 1 1.5 h after injection. A rapid elimination followed, the level of activity being 3.5 ± 0 mg per 1 3.5 h after injection, 0.5 ± 0.5 mg per 1 at 7 h and less than 0.4 mg per 1 at 15 and 24 h. A single injection of 15 mg MDZ resulted in a mean serum level of 24 ± 3.2 mg per 1 1.5 h after injection. After 3.5 h the level had fallen to 7.9 ± 2.9 mg per 1 and after 7 h to 3.8 ± 1.3 mg per 1. No MDZ activity was detectable at 15 and 24 h (less than 0.4 mg per 1).

The serum half-life of MDZ in the guinea-pig was approximately 3 (3.0 to 3.5) h.

Response of experimental B. fragilis otitis media to MDZ treatment (III). A total of 59 guinea-pigs were challenged with 0.5-1.0x10⁸ cfu of B. fragilis and a seven days course of MDZ treatment was initiated on day 7 after challenge. All animals were sacrificed on day 14 after challenge and their middle ears analyzed. Ip injection of 6 mg MDZ (about 20 mg per kg) once daily, 6 mg twice daily and 15 mg (50 mg per kg) once daily reduced the incidence of culture-positive ears from 13/17 among untreated controls to 7/17 (p = 0.08) 3/10 (p = 0.05) and 2/15 (p = 0.001), respectively.

Serum antibody response to B. fragilis, P. acnes and P.anaerobius (IV). Twentythree guinea-pigs challenged with B. fragilis and sacrificed on day 5 (n = 3), day 10 - 12 (n = 12) or day 21 (n = 8) were randomly selected from a total of 44 animals (II). IgG antibodies versus the challenge organism were demonstrated in 10 of the 23 sera drawn before challenge. IgM antibodies were demonstrated in 4 of these sera and IgA antibodies in only one of them. A significant increase (>4-fold) of IgG and IgM titres was noted in 1/3 sera obtained on day 5 after challenge. On day 10 - 12 9/12 sera showed increased IgG and 8/12 sera contained increased IgM titres. Five and 4 sera out of 8 showed significant increases of IgG and IgM titres respectively on day 21 after challenge. Elevated IgA antibody titres were demonstrated on day 21 in 2/8 sera. Neither P. acnes nor P. anaerobius (12 and 7 paired sera, respectively) had induced a serum antibody response in the guinea-pig on day 5, 10 or 12 after challenge.
INTERACTION BETWEEN PMNS AND BACTERIA

Effect of serum components on aerobic phagocytosis and killing of *P. acnes* (V). When incubated aerobically in buffer in the absence of serum and leukocytes, the number of viable *P. acnes* organisms was essentially unchanged for at least 60 min at 37°C. In the presence of 5 % PNHS, a minor (15 - 20 %) reduction of the number of viable bacteria occurred and incubation with leukocytes in the absence of serum resulted in phagocytosis and killing of about 70 % of the *P. acnes* cells during 60 min. In the presence of PMNs and 5 % PNHS more than 99 % of the bacteria were phagocytosed and 98 % were killed within 30 min with only slight additional killing occurring during the next 30 min. Also human serum depleted of IgG and IgM efficiently promoted phagocytosis of *P. acnes*. In contrast, serum that had been heat-inactivated, depleted of complement component C3, or depleted of C4 and factor B, did not promote phagocytosis or killing. PNHS depleted of C4 or factor B still had unimpaired capacity to promote phagocytosis of *P. acnes*. These results indicated that phagocytosis of *P. acnes* is mediated by complement component C3, activated either by the classical or by the alternative pathway.

Functional control of anaerobiosis (V and VI). Phagocytosis of *S. aureus* was equally efficient under aerobic and anaerobic conditions, whereas killing was considerably delayed in the latter case. Thus, 0 - 30 % of the staphylococci were killed during anaerobiosis as compared to more than 80 % in air, indicating that O₂ dependent mechanisms cidal to *S. aureus* were indeed impaired in our anaerobic experiments. *S. faecalis* was included in the experiments to further check the cidal power of PMNs during anaerobiosis (Mandell 1974). As expected, more than 98 % of the *S. faecalis* cells were phagocytosed and killed under aerobic as well as anaerobic conditions (V and IV).

Role of oxygen in phagocytosis and killing of *P. acnes* (V). In the absence of serum, phagocytosis was as efficient under anaerobiosis as in air (70 %, see above). However, only half of the phagocytosed bacteria were killed under anaerobiosis as compared to all *P. acnes* cells in air. Also in the presence of 5 % PNHS,
anaerobiosis reduced killing (but not phagocytosis) of *P. acnes* (NCTC 737 and MC-7). Thus, under anaerobiosis only 50% of the bacteria were killed as opposed to 98% in air.

**Role of oxygen in phagocytosis and killing of *S. pneumoniae* (VI).**

Encapsulated pneumococci of serotypes 9N, 14, 21 and 23F were efficiently phagocytosed in the presence of 10% PNHS under aerobic as well as anaerobic conditions. For efficient phagocytosis of encapsulated type 2 pneumococci 20% immune serum was required and for purpose of comparison this serum supplement was used also in experiments with unencapsulated type 2 pneumococci. None of these serum supplements were cidal to any of the bacterial strains studied.

Aerobic phagocytosis was instantly followed by killing of the pneumococci irrespective of serotype and for type 2 irrespective of the presence or absence of capsule. Thus, 96–99.6% of the pneumococci were phagocytosed and killed during a 60 min incubation together with PMNs and serum. Under anaerobic conditions killing of the pneumococci was considerably delayed, (70–85% killing at 60 min). The presence of type 2 capsule did not confer the pneumococci any resistance to killing after phagocytosis. Using a defined growth medium to obtain autolysis resistant pneumococcal cells (see Materials and methods) it was found that the pneumococcal autolytic system did not significantly contribute to their aerobic or anaerobic killing by human PMNs.

**Degradation of *S. pneumoniae* macromolecules by human PMNs (VI).**

The fate of specifically isotope labelled encapsulated serotype 14 *S. pneumoniae* (teichoic acid, unsaturated cytoplasmic cell membrane lipids, DNA and RNA were separately labelled) after phagocytosis was studied. Degradation of bacterial cell wall material and intracellular macromolecules (DNA and RNA) proceeded almost as efficiently during anaerobiosis as in aerobic phagocytosis. However, the cytoplasmatic membrane suffered from considerable losses of lipid radioactivity only during incubation in air. Autolysis resistant pneumococci were degraded to the same extent as normal autolytic pneumococci. Thus, type 14 pneumococci were killed (see above) and degraded under aerobic and anaerobic conditions due to the action of PMN without the aid of the bacterial autolytic system. Since pneumococcal DNA and RNA were degraded by anaerobic PMNs despite little loss of bacterial lipid radioactivity different
mechanisms of damage to bacterial membranes during anaerobic killing and aerobic killing by PMNs were suggested.
GENERAL DISCUSSION

Animal models for experimental otitis media. Otitis media, defined as an inflammation of the mucosa of the middle ear (Paparella 1976) is easily induced in guinea-pigs, rabbits, chinchillas and gerbils by administration of suspensions of bacteria or bacterial products into the middle ear cavity via the tympanic membrane or by trans-bulla injection (Haymann 1912-1913, Juhn et al 1977, Lowell and Juhn 1979, Lowell et al 1980). Experimental otitis media has further been induced after nasal inoculation of pneumococci (Haymann 1912-1913, Giebink et al 1979, 1980).

The guinea-pig middle ear is sensitive to a variety of pyogenic bacteria, i.e. Streptococcus pyogenes, S. pneumoniae, S. aureus, Proteus species and Pseudomonas aeruginosa (Haymann 1912-1913, Friedmann 1955, Lim and Klainer 1971). Bacterial challenge may cause ciliostasis of the mucosa, oedema of the subepithelial space with infiltration of PMNs, followed by macrophages and plasma cells. Bacteria may persist for several months in the middle ear cavity. Healing occurs through fibrosis of the subepithelium. New bone formation (often heavy with final obliteration of the middle ear cavity) may occur. The acute phase of the infection is sometimes accompanied by labyrinthitis, septicaemia, meningitis and abscess formation in the brain and cerebellum and eventually death of the animal.

Little information regarding the behaviour of anaerobic bacteria in experimental otitis media is available. Mixtures of aerobic and anaerobic bacteria can however produce intense and even necrotizing otitis in rabbits or gerbils (Wirth 1935, Fulghum et al 1982). Thus, anaerobic bacteria may participate in aggressive middle ear infections in apparent cooperation with other bacteria.

The present animal model. While workers so far have dealt with the possible role of anaerobic bacteria in mixed middle ear infections (see further below), the present study showed that using high doses of P. acnes or B. fragilis anaerobic monoinfections can be established in the middle ear of guinea-pigs. Fulghum and coworkers (1982) reported analogous results for P. acnes in experi-
ments with gerbils for induction of anaerobic monoinfections of the middle ear.

Gentamicin prophylaxis was used in all animal experiments in the present investigation. This agent may enhance the virulence of \textit{B. fragilis} in experimental infections in rabbits (Rotilie et al 1974), but preliminary experiments did not suggest such gentamicin effects in our model.

The anaerobic infections here established were at the early stages histologically similar to the experimental pneumococcal otitis media (I), and the two anaerobic species induced new bone formation. The \textit{P. acnes} infection subsided within 10 days leaving minute new bone as sequela, whereas the \textit{B. fragilis} infection was more protracted and bacteria could be isolated from the middle ear up to 21 days after challenge. The periostal bone reaction to \textit{B. fragilis} was more pronounced than that seen after \textit{P. acnes} challenge, but not as marked as reported for strains of pneumococci and \textit{Pseudomonas} species in the guinea-pig middle ear (Haymann 1912-1913, Friedmann 1955). None of our animals died in generalized infection upon challenge with anaerobic bacteria and not even labyrinthitis occurred. This may reflect the inferior virulence of anaerobes as compared to pneumococci as shown in other studies of experimental otitis media using guinea-pigs (Haymann 1912-1913, Friedmann 1955).

The capacity of the type 23 pneumococcal strain here used to produce a standard otitis media was only slightly superior to that of \textit{P. acnes} (I). However, in chinchilla otitis experiments type 23 pneumococci were found to be of considerably lower virulence than were type 3 pneumococci (Giebink et al 1977, Watanabe et al 1982). It is possible that this difference between pneumococci is true also in guinea-pigs making the type 23 pneumococcal strain less suitable for comparisons of the virulence between anaerobic and aerobic bacteria in our model.

Since anaerobes here were able to induce a monoinfection in the middle ear of healthy guinea-pigs our data support the contention that such bacteria play an active role in middle ear infections provided that they get a chance to invade. It is thus likely that
anaerobes truly contribute to the infectious process in COM where they are the only organisms isolated in 10% of the cases (Finegold 1981). They may also be an occasional cause of AOM as suggested by Brook (1979). This hypothesis of course needs to be investigated in future studies.

Pathogenesis of anaerobic infections. Our observation that *B. fragilis* is capable of inducing otitis media in the guinea-pig is not surprising since it has been shown that *B. fragilis* isolated from COM is highly virulent producing generalized disease and abscesses upon injection into guinea-pigs (Hudac et al. 1983). The ability to adhere to epithelial surfaces seems to be an important first-stage virulence factor in many bacteria, and *B. fragilis* and strains of pigmented *Bacteroides* species can adhere to epithelial surfaces (Onderdonk et al. 1978, Okuda et al. 1981). Furthermore, *B. fragilis* can multiply in the presence of air on the surface of rabbit tracheal membrane explants and cause ciliestasis, or in monolayer tissue cultures (Matsuyama 1974, Murakami and Matsuyama 1980, Murakami et al. 1981, Matsuyama et al. 1982). The low O₂ tension in the explants and the cell cultures due to the metabolic activities of the mammalian cells enabled growth of the anaerobe.

Other animal experiments have shown that anaerobic bacteria, notably *B. fragilis* may cause monoinfections such as intraabdominal, liver and subcutaneous abscesses and establish themselves in subcutaneously implanted tissue-cages (Kasper et al. 1977, Onderdonk et al. 1977, Wilkins et al. 1977, Tonjum et al. 1979, Rylander et al. 1981, Kasper and Onderdonk 1982, Schwan and Danielsson 1983). The capacity of *B. fragilis* to cause abscesses has been attributed to the presence of a polysaccharide capsule, and unencapsulated related species are harmless in animals. Similarly, subcutaneous abscesses in guinea-pigs and rabbits have been induced with an encapsulated strain of *B. melaninogenicus* (Takazoe et al. 1971). The production of collagenase has been suggested to be another virulence factor of *B. melaninogenicus* (Smith 1975).

The brain of the rat is susceptible to challenge with high doses of *B. fragilis* but not with anaerobic Gram positive cocci such as *S. intermedius*, *P. anaerobius*, *P. asaccharolyticus* or
P. prevotii (Costello et al 1983). In general, anaerobic Gram positive cocci as well as B. asaccharolyticus seem to be of low virulence in various animals (Smith 1975).

This may be the reason for the shortcoming of such species in our otitis model. P. acnes, a Gram positive rod shaped bacterium may induce severe inflammation upon intradermal injection in rabbits, and its production of hyaluronidase, neuraminidase and lipase have been suggested as possible virulence factors (Smith, 1975). Hudac and coworkers (1983) reported that a strain of P. acnes isolated from COM showed moderate virulence upon im injection in guinea-pigs forming i.e. intraabdominal abscesses.

Since anaerobic bacteria are often isolated together with aerobic bacteria in human infections (this is also the case in COM), several animal models have been used to study the role of anaerobic bacteria in mixed infections. Experimental mixed infections involving aerobic and anaerobic bacteria have been induced in the middle ear (see above) and also in other parts of the respiratory tract, i.e. in the lungs of the rat. B. fragilis seems to be essential for induction of such experimental infections, and the importance of using fresh isolates has been stressed (Kannangara et al 1981, File et al 1982).

The mechanisms of the apparent synergism between aerobic and anaerobic bacteria have been clarified in a few instances. For example, Fildes (1929) demonstrated that tetanus spores would not germinate when inoculated alone subcutaneously into guinea-pigs. It was later demonstrated that when aerobic bacteria were introduced concurrently, the redox potential was reduced (indicating a fall in local pO2) permitting the tetanus spores to germinate (Woods and Foster 1964, quoted by Gorbach and Bartlett 1974). Similarly, B. melaninogenicus is often avirulent in experimental infections unless vitamin K is supplied by certain aerobic bacteria (McDonald et al 1960).

Synergy between e.g. B. fragilis and E. coli has clearly been demonstrated in experimental animal models for intraabdominal sepsis and wound infection (Onderdonk et al 1974, 1976, Kelly 1980). Also, synergy between different anaerobic bacterial species has been shown in animal experiments (Hite et al 1949, Socransky and Gibbons 1965, Sundqvist et al 1979).
It has been suggested that in human maxillary sinusitis anaerobic bacteria are secondary invaders helped by an O\textsubscript{2}-free milieu established by aerobic bacteria at an early stage of the infection (Frederick and Braude 1974). Furthermore, \textit{in vitro} experiments have shown that several aerobic bacterial species including pneumococci, cultured in broth tend to deplete the medium of O\textsubscript{2} regardless if O\textsubscript{2} is utilized by the bacterial metabolism (Jacob 1970, Carenfelt and Lundberg 1978). Such mechanisms may operate in COM, helping anaerobic opportunistic bacteria to invade the middle ear and contribute to the persistence and dissemination of the infection.

\textbf{MDZ in otitis media.} Anaerobic infections occur not only in the middle ear but also at other sites of the respiratory tract in humans. Thus, acute and chronic sinusitis and aspiration pneumonia may be due to anaerobic bacteria (Bartlett et al 1974, Frederick and Braude 1974, van Cauwenberge et al 1976, Lundberg et al 1979, Bartlett 1981, Collee 1982), and anaerobic mixed infection of the tonsils or the paranasal sinuses have successfully been treated with nitroimidazoles (Shinn 1962, Davies et al 1964, Lundberg et al 1981). Nitroimidazoles have also been useful in the treatment of intracranial complications and bacteraemia secondary to COM (Ingham et al 1977, Beeden and Willis 1980, Siegler et al 1982). Since the potential danger in COM is the risk of local or metastatic spread of the infection, systemic nitroimidazoles may seem to be a reasonable choice of additive regimen also in COM with anaerobic bacteria or when complications are suspected (see also Introduction). However, Browning and coworkers (1983) found that MDZ administered to humans in high doses was not sufficient to efficiently eliminate middle ear anaerobes in COM unless combined with other antibiotics. Similarly, Moloy (1982) failed to cure a case of massive \textit{B. fragilis} mastoiditis with MDZ.
The present investigation demonstrated that systemically administered MDZ accelerated the elimination of *B. fragilis* from the middle ear of the guinea-pig, although not even high doses of the drug were fully effective. Sufficient local concentrations of MDZ were probably achieved, since the serum levels widely exceeded the MIC for the *B. fragilis* strain used (0.8 mg/l, III) and since in COM, MDZ doses lower than those used here have been shown to produce MEE MDZ levels well above those required to inhibit most anaerobic bacteria (Jokipii and Jokipii 1981). The failures may instead reflect an incomplete anaerobiosis at the site of infection in some instances, since it has been shown in vitro that MDZ is dependent in strict anaerobiosis for killing *B. fragilis*. Even small quantities of O₂ not impairing the viability of the anaerobe may cause conditional MDZ resistance in susceptible organisms (Milne et al 1978). At present, nitroimidazoles in COM must therefore be regarded as a possible therapeutic alternative requiring further study, particularly with regard to the dosage.

The humoral immune response The role of serum antibodies in otitis media has earlier been experimentally investigated in chinchilla models for pneumococcal otitis. Thus, whereas type 23 pneumococci evoked a serum antibody response the type 3 pneumococci stimulated the local production of antibody in the chinchilla middle ear (Giebink et al 1977, Watanabe et al 1982b). The type 23 infection was self-lmiting, whereas the type 3 infection had a prolonged course. These studies suggested that a raise in serum antibody versus the infecting pneumococci mirrored the elimination of bacteria from the middle ear and that serum antibodies may play an important role in dictating the clinical course of pneumococcal otitis media. The importance of serum antibodies was further supported by experimental studies in chinchillas where systemic vaccination with pneumococci was found to induce serum antibodies versus the homologous pneumococcal strain protective to otitis media following nasal colonization (Giebink et al 1979). Also in human AOM serum antibodies are of importance for the prevention of the infection (see Introduction).

In the present otitis model the humoral immune response to *B. fragilis*, *P. acnes*, and *P. anaerobius* was studied. Using IFL technique, induction of serum antibody against the *B. fragilis* strain
but not against \textit{P. acnes} or \textit{P. anaerobius} was demonstrated. \textit{B. fragilis} induced an IgG and IgM response (but almost never IgA) as found also in experimental type 23 pneumococcal otitis media in chinchillas (see above).

A two-phased growth curve and the induction of a humoral immune response and a local inflammatory reaction including new bone formation, indicated that \textit{B. fragilis} caused true otitis media in the guinea-pig rather than a temporary survival upon challenge. The lack of antibody response to \textit{Peptostreptococcus anaerobius} is not surprising, since no inflammation was induced with that strain. \textit{P. acnes} induced an inflammation although this subsided within 10 days. No antibodies against the strain could be demonstrated although the inflammation was accompanied by an accumulation of PMNs in the subepithelial space – an evidence of chemotaxis. \textit{P. acnes} infections in humans may induce antibodies (Webster et al 1980), and the lack of such a response in our model may be interpreted as an expression of the rather low virulence of this organism in the guinea-pig middle ear.

In otitis media in humans, the possible immune response to anaerobic bacteria has never been considered, although Hudac and coworkers (1983) reported that low titres of antibodies against anaerobic bacteria isolated from COM could be demonstrated in single serum specimens at the time of surgical intervention. Their findings are in agreement with other studies showing that anaerobic bacteria may induce a humoral immune response in humans (Lambe et al 1975, Kasper et al 1978, Rissing et al 1979, Falkler et al 1982). Thus, \textit{B. fragilis} infections (septicaemia, pyothorax, pelvic inflammatory disease) evoke a strong serum IgG response versus capsule polysaccharide and lipopolysaccharide and \textit{Fusobacterium nucleatum} may probably induce IgG and IgA (but not IgM) responses in chronic periodontitis (Falkler et al 1982). The humoral immune response to \textit{B. fragilis} in animal models have been studied, and the capsular polysaccharide was shown to be responsible for the induction of specific IgG and IgM antibodies (Kasper et al 1977, Ellis and Barrett 1982, Kasper and Onderdonk 1982, Schwan and Danielsson 1983).

IgG and IgM antibodies are actively involved in phagocytosis, and
the induction of opsonizing antibodies has been demonstrated in experimental *B. fragilis* infection (Kasper och Onderdonk 1982, Ellis and Barrett 1982). Furthermore, anticapsular (as for *S. pneumoniae*, see Introduction) or antilipopolysaccharide antibodies as well as complement have been shown to be necessary for phagocytosis of encapsulated *B. fragilis* by PMNs (Bjornson et al 1976, Bjornson and Bjornson 1978, Simon et al 1982). Although the induction of opsonizing antibodies against *B. fragilis* was not considered in the present investigation, it is likely that the increased anti *B. fragilis* titres observed are an expression of an increased opsonizing capacity of the guinea-pig serum involved in limiting the *B. fragilis* otitis media.

Several studies have shown that anaerobic bacteria, including *P. acnes*, can induce chemotaxis (Hofstad and Sveen 1979, Sundqvist and Johansson 1980, Lee et al 1982). The chemotactic agent C5a is formed during complement activation, and with *P. acnes* such activation may take place without the aid of antibodies (McBride et al 1975, Massey et al 1978, Okuda et al 1978). Furthermore, the present investigation demonstrated that, provided complement is present, efficient phagocytosis of *P. acnes* can take place in vitro without the aid of antibodies (V). Together with the observation that PMNs accumulated in the middle ear mucosa upon challenge with *P. acnes* (I) this suggested that PMNs to some degree contributed to the elimination of *P. acnes* from the guinea-pig middle ear (see further below).

*O₂* tension of MEEs. The killing of many microorganisms by PMNs is dependent on the presence of *O₂* (see Introduction). In SOM, MOM and CSOM the *O₂* tension of the MEE is lower (but without approaching anaerobiosis) than the *O₂* tension of the air of the middle ear cavity under normal conditions (Drettner 1975, Ingelstedt et al 1975, Ostfeld et al 1980). The supply of *O₂* to the middle ear mucosa may be much reduced during disturbances of the microcirculation after eustachian tube occlusion although the *pO₂* of the air in the middle ear cavity is only slightly reduced (Matsumura 1955, Morgenstern 1980). In contrast, complete anaerobiosis is typical of the purulent effusions in acute maxillary sinusitis, in pleural empyema and in experimental abscesses in rats due to staphylococci or *E. coli* (Funahasi et al 1971, Hays
and Mandell 1974, Carenfelt and Lundberg 1977, 1978). Although little information is available regarding the $O_2$ tension of the purulent MEEs in AOM and COM, a milieu allowing growth of obligate anaerobic bacteria is probably depleted of $O_2$. It is likely that in AOM the local environment often becomes anaerobic since exponentially growing pneumococci (and the PMNs accumulated) may deplete the MEE of $O_2$. The effect of MDZ in experimental B. fragilis otitis in the present investigation is another indirect evidence of a low $O_2$ tension in purulent MEE.

Interaction between anaerobic bacteria and PMNs. B. fragilis, P. anaerobius (studied here in the otitis model), B. thetaiotaomicron, Fusobacterium mortiferum and Clostridium perfringens are efficiently killed by human PMNs regardless of the presence or absence of $O_2$ (Mandell 1974, Bjornson et al. 1976, Bjornson and Bjornson 1978). In contrast, we found that killing of P. acnes by human PMNs was inefficient under anaerobic conditions (V). Since this organism is sometimes found in COM it is tempting to speculate that it is maintained in the pus because it survives phagocytosis. The presence of P. acnes may continue to induce chemotaxis in PMNs which as we have observed accumulate at the site of infection. The PMNs contribute to a low local $O_2$ tension (and further growth of P. acnes) and to tissue damage by their proteolytic enzymes (Kastenbauer et al. 1975, Webster et al. 1980, Carlsson et al. 1982, Wright 1982, Engqvist et al. 1983). Thus, despite its relatively low virulence, P. acnes rather than being only a contaminant from the external ear canal may play an important role in maintaining an otitis media through the hypothetical vicious circle outlined above.

Another interesting phenomenon in the interaction between anaerobic bacteria and PMNs has been demonstrated by Ingham and coworkers (1977) who reported that B. melaninogenicus, the most commonly isolated anaerobic species in COM, inhibited phagocytosis of aerobic bacteria by some hitherto unknown mechanism. Such interaction with host defence further suggests an intriguing pathogenic role of the anaerobic component of the mixed flora in COM.
Interaction between S. pneumoniae and PMNs. Although there is direct evidence of an anaerobic milieu in maxillary sinus empyema and circumstantial evidence of such conditions in otitis media, it is surprising that the interaction between the most common pathogen in AOM, S. pneumoniae, and human PMNs under anaerobic conditions has not been studied earlier. In the presence of air encapsulated pneumococci are readily phagocytosed by PMNs provided they are properly opsonized by immunoglobulin and complement (see Introduction). They are rapidly killed by the phagocytes under aerobic conditions, and peroxide, generated by the PMNs or the bacteria, seems to be essential for optimal killing (Pitt and Bernheimer 1974). A possible cause of the cidal effect of PMNs is peroxidation of pneumococcal unsaturated cytoplasmatic membrane lipids (Shohet et al 1974). Other pneumococcal macromolecules are attacked by degrading PMN enzymes (see Introduction) whereas the pneumococcal autolytic system which is assumed to play an important role in the cidal effect of betalactam antibiotics, does not seem to be involved in their degradation by aerobic PMNs (Tomasz et al 1977).

Pneumococci were found to be phagocytosed and killed by PMNs also under anaerobic conditions (VI). In comparison with aerobic conditions anaerobic killing was, however, markedly retarded, suggesting that O₂-dependent killing mechanisms of PMNs significantly contributed to killing of pneumococci by aerobic PMNs. Since the autolytic system of the pneumococci did not contribute to the process anaerobic killing was solely due to the action of as yet unknown O₂-independent PMN functions.

Killing was followed by partial degradation of pneumococcal cell wall teichoic acid, deoxyribonucleic and ribonucleic acids under aerobic as well as anaerobic conditions but not accompanied by any significant degradation of pneumococcal cytoplasmic unsaturated lipids under anaerobiosis. Thus, other membrane damaging PMN functions not requiring O₂ then seemed to open the pneumococcal cell. These O₂ independent bacteriolytic mechanisms remain to be analyzed in future studies.

In conclusion, the present studies of host defence in otitis have shown that the virulent pneumococcus is quite easily handled by the PMN cell irrespective of the presence or absence of O₂. The
Achilles' heel in the defence against this bacterium seems to be the obligate requirement of specific antibodies and complement which plays an important role in pneumococcal otitis media. In contrast, the less virulent *P. acnes* is easily phagocytosed by PMNs and the host is not dependent on antibodies for this process. However, under anaerobic conditions *P. acnes* is inefficiently handled by PMNs, a feature which makes it unusual among anaerobic bacteria (see above) and also different from the pneumococcus. This behaviour of *P. acnes* and the anaerobic conditions presumed to be present in COM may help *P. acnes* to maintain the inflammation of the middle ear via the vicious circle postulated above.
SUMMARY AND CONCLUSIONS

I. A guinea-pig model for induction of otitis media with different species of anaerobic bacteria was established. Using this model it was shown

a. that *B. fragilis* and to a lesser extent *P. acnes* induced a typical acute otitis media, whereas *B. asaccharolyticus*, *P. anaerobius* and *P. micros* failed to induce otitis media in the guinea-pig.

b. that the establishment of an anaerobic monoinfection was dependent on a high challenge dose, and that the infection was self-limiting, suggesting that anaerobic bacteria alone are of relatively low virulence in the guinea-pig middle ear.

c. that metronidazole accelerated the elimination of *B. fragilis* from the middle ear.

d. that *B. fragilis* but not *P. acnes* or *P. anaerobius* induced a humoral antibody response, suggesting true virulence of *B. fragilis* in guinea-pig middle ear monoinfections.

The results of the present study support the contention that anaerobic bacteria are involved in otitis media as true pathogens. As a consequence and due to the risk of anaerobic complication, especially of the CNS, an additional antimicrobial regimen directed against the anaerobic flora in COM is advocated.

II. An *in vitro* model for studies of the interaction between human PMNs and bacteria with special reference to anaerobiosis was established. Using this model it was shown

a. that *P. acnes* was readily phagocytosed by human PMNs with the aid of C3 activated either via the classical or the alternative pathway.

b. that killing of *P. acnes* by PMNs was inefficient during anaerobic conditions, providing a basis for a model proposed to
describe a possible pathogenic role of this microorganism in COM.

c. that *S. pneumoniae* was killed by PMNs under anaerobic conditions although at a considerably slower rate than in the presence of air.

d. that degradation of pneumococcal teichoic, deoxyribonucleic and ribonucleic acids took place after phagocytosis under aerobic as well as anaerobic conditions, whereas degradation of unsaturated cell membrane lipids took place only under aerobic conditions.

e. that the pneumococcal autolytic system did not participate in the killing or the degradation of the bacteria by human PMNs.
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