ABSTRACT

Mark E. Beamer. EXPERIMENTAL OTITIS MEDIA IN GERBILS USING SINGLE AND POLYMICROBIAL ANAEROBIC BACTERIAL CULTURES. (Under the direction of Dr's. Wendell E. Allen and Robert S. Fulghum) Department of Biology, September 1985.

The objective was to determine pathogenic potentials of anaerobic bacteria and known mixtures of anaerobic bacteria to cause otitis media. Gerbils were inoculated per cutaneously into the middle ear bulla with 4.5×10^6 bacteria per ear. Evaluation was by ototoscopic and histologic observations. Nine different anaerobes and one facultatively anaerobic bacterium used were: Peptococcus magnus, Peptostreptococcus anaerobius, Ps. intermedius, Propionibacterium acnes, Gaffyka anaerobius, Clostridium perfringens, Bacteroides fragilis, B. melaninogenicus, Fusobacterium nucleatum and Escherichia coli. The ability of both living and dead cultures, as well as mixed cultures, to cause otitis media was compared using a predetermined scoring system. The time course and sequellae (persistant granulation tissue, bone damage, and new bone formation) of the otitis media were similarly compared for a period of up to 4 weeks. Inflammatory responses with no sequellae were produced by 2 of the pure cultures but none of the mixtures. Moderate otitis media was caused by 6 of the pure cultures and 7 of the mixtures. Severe otitis media with granulation tissue and remarkable bone changes were found caused by 2 of the pure cultures and 5 of the mixtures. Synergy appeared to be responsible for more severe otitis media with 5 of the mixtures as compared to the response to the individual pure cultures of the mixture. We conclude that experimental otitis media can be caused by anaerobic bacteria in the gerbil. In humans, natural otitis media caused by or contributed to by anaerobes probably occurs as secondary infections following acute otitis media during which conditions for anaerobes to grow are extablished.

EXPERIMENTAL OTITIS MEDIA IN GERBILS USING SINGLE AND POLYMICROBIAL ANAEROBIC BACTERIAL CULTURES

A THESIS

PRESENTED TO THE FACULTY AND DEPARTMENT OF

BIOLOGY

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Mark Edward Beamer

July, 1985

EXPERIMENTAL OTITIS MEDIA IN GERBILS USING SINGLE AND POLYMICROBIAL ANAEROBIC BACTERIAL CULTURES

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ACKNOWLEDGEMENTS

I would like to acknowledge the contribution of Dr. Robert Fulghum toward the successful completion of this thesis. The use of his lab, participation, experience, and insight have all contributed to make this work possible.

I would also like to thank: Dr's. Henry Marrow and Jack Brinn for their advice in evaluating the slides; Mr. John Worthington for his help in inoculating and sacrificing animals, identifying bacteria, and preparing the media used in these experiments.

I would like to thank Dr's. Wendell E. Allen and Charles O'Rear for their time and effort while serving on my committee.

Finally, I would like to thank my wife for all her work in typing and retyping the manuscript, and support which helped me through.

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Introduction

Otitis media is a general term for a variety of pathological disorders of the middle ear cleft. These disorders have many etiologies: anatomical, physiological, and microbial. This paper will discuss otitis media of bacteriological origin with specific emphasis on anaerobic bacteria. Otitis media of microbial origin is generally an inflammation of the mucoperiosteal lining of the middle ear. There are numerous complications of this disease. Otitis media is also the leading cause of conductive and sensorineural hearing loss (Rapin,1979). Due to the seriousness of the disease, and the potential of life threatening complications associated with the disease, research into the etiology and treatment of this disease is invaluable. Many individuals are predisposed to otitic infection because of certain physiological, immunological, and anatomical conditions. Many of the microorganisms associated with the disease are anaerobic; therefore, study in this area is especially pertinent since antibiotic treatment not aimed at the specific organism(s) involved is futile (Fulghum et al. 1977).

REVIEW OF LITERATURE

History

There are many early references to otitis media. In 1881, Bezold and Siebermann described complications resulting from chronic cases of otitis media (Surkin et al. 1983). These cases involved infection of the mastoid and the sternomastoid muscle. In 1898, Rist made reference to anaerobic bacteria found in suppurative disease of the mastoid and middle ear. Krumwiede and Pratt, in 1913, isolated anaerobic bacilli and spirochetes from ear discharges. In 1923, Busacca outlined ten cases of chronic suppurative otitis media that were highly similar to anaerobic and spirochetal infection. It is now realized that anaerobic bacteria play a significant role in otitis media of pure and polymicrobial infection. Therefore, it is speculated that many of the earlier specimens that were not shown to contain anaerobes could possibly have been anaerobic infections that were not detected due to improper specimen collection and transportation.

Sugita et al. (1983) documented the difference in culture collection techniques in microbiological studies of otitis media in pre-world war II Japan. He showed Haemophilus influenzae as the major causitive agent and Staphylococcus aureus being infrequently isolated, the opposite of what was found in the United States and Scandinavia. Sugita and coworkers proposed that differences in the culture techniques were

causing the discrepancy. In Japan, chocolate agar (needed for the isolation of Haemophilus influenzae) was used routinely in cultures, while it was not used in the United States and Scandinavia. Other technique differences included the use of post acute phase samples, and failure to totally disinfect the ear canal and external meatus prior to sample collection.

Anaerobic Environment

It is generalized that approximately 40% of all cases of otitis media contain anaerobes (Brook,1980). Anaerobic bacteria may account for discrepencies in previous literature regarding bacteria types and possible sterile specimens because they were not cultured anaerobically. Many conditions in the middle ear make it a favorable environment for the growth of anaerobic bacteria. In this case, anaerobic may be a misleading term. Anaerobic bacteria possess a wide range of oxygen tolerance spanning from facultative anaerobes to strict anaerobes. Other factors that play a part in the growth and survival of anaerobic bacteria are the abilities of the organism to produce oxidases, catalases and super oxide dismutase along with the ability to transfer electrons metabolically at various oxidation-reduction potentials (Fulghum et al. 1977).

Conditions in the Middle Ear

Giebink and Quie (1978) pointed out that there are numerous factors

that affect the middle ear and its ability to harbor and facilitate growth of different species of anaerobic bacteria. The eustachian tube functions in the ventilation of the middle ear. These studies on tubal malfunction in children confirmed the relationship between this and chronic otitis media in children. They concluded that pressure equalization (yawning, deglutition, nose blowing) aided in ventilation of the middle ear which allowed gas exchange and maintained a fairly aerobic environment. They also found that the reverse is true. A blocked eustachian tube leads to a pressure decrease inside the middle ear resulting in the transudation of fluid into the middle ear in order to equalize pressure. This equalization of pressure by decreasing the air pressure inside the middle ear forms a facultative anaerobic environment (Giebink and Quie, 1978). Along with the blockage induced anaerobic environment, other conditions for the growth of anaerobes are encountered. Biochemical studies showed increased oxidative and hydrolytic enzyme activity throughout the mucoperiosteum due to a widening of the subepithelial space from the accumulation of fluid (Giebink and Quie, 1978). In summary, their results have shown that there are variances in the oxidation-reduction potential inside the middle ear, and also variation in anaerobic needs of the bacteria themselves. Due to these changes in the middle ear and differences among the bacteria themselves, the middle ear may form a hospitable site for the growth and development of anaerobic bacteia, even though it does contain some oxygen.

Clinical Significance of Anaerobic Bacteria

Chronic otitis media in children is insidious, persistent, and destructive with such irreversible sequelae as hearing deficit, learning disabilities, balance disorders and many more complications (Brook, 1980). In pre-antibiotic and chemotherapeutic times, chronic otitis media was a dangerous disease with a high mortality and complication rate (Table I). These complications are mostly intracranial and consist of cerebal abscess, meningitis, sinal thrombosis, otitic hydrocephalus, and cortical thrombophlebitis. The mortality rate has since declined but is still clinically signiticant (Pfaltz, 1982). Extracranial complications that affect the facial nerves and eustachian tube may cause deafness, balance dysfunction, and facial palsy (Pfaltz, 1982).

Brain abscesses, often otogenic in nature, suggest that either anaerobic bacteria penetrate the brain more readily than aerobic bacteria, or they are more common in chronic otitis media than the literature proposes (Brook, 1980). Present research shows the latter theory to be true. Brook (1980) obtained 108 aspirates from 68 patients with otitis media having perforation of the tympanic membrane. Forty of the patients had bilateral aspirations and all had previously been treated with antimicrobial therapy. The organisms obtained were considered to be true pathogens since aspirations were performed from both the middle ear cleft and external canal. Brook preferred tympaniocentesis, aspiration through the tympanic membrane, because of decreased risk of contamination. Organisms that were in the auditory canal and not the middle ear were considered contaminants. The overall results of this study showed that 51% of all infected ears were either

anaerobic or aerobic with anaerobic mixtures in the infection (Table II). Jokippi and coworkers (1977) reported that of 70 cases, 50% of the anaerobes isolated were Bacteroides species . They found that anaerobes were most always a part of a mixed flora infection, mostly as two anaerobes and two aerobes per infection and theorized that microbial synergy was the reason for these mixtures. This may occur by reducing the oxidation-reduction potential inside he middle ear or destruction of the tissue.

In early studies, anaerobes were not considered a major cause of chronic otitis media. Recent studies have shown increased resistance to penicillins and aminoglycosides in anaerobes. These antibiotics, when used against the aerobes in the infection, left the anaerobes unharmed and with less competiton. Many of the ear drops on the market today contain neomycin and/or gentamycin to which many of the anaerobes are resistant (Jokippi et al. 1977).

Sugita et al. (1981) studied the prevalence of polymicrobial anaerobic infection. Of 62 anaerobic infections, 75.4% contained one anaerobic specimen, 19.7% contained two different species, 3.3% contained three different species, and 3.3% contained four different species (table III). Cholesteatoma has been suspected in providing a hospitable atmosphere for anaerobes by blocking the eustachian tube, thus, interferring with drainage. This is most likely a major factor, because 54% of all cases with anaerobic infection showed presence of cholesteatoma (Sugita et al. 1981).

Although all ages are susceptible to otitis media, children are often predisposed to the condition (Giebink and Quie,1978). It has been

shown that 90% of children may be affected at some point in their childhood (Giebink and Quie, 1978). The prevalence of otitis media in present society was described by the British Medical Research Council in 1957. Out of 47,500 individuals, 1162 (2.4%) had at least one episode of otitis media in a 1 year period (Giebink and Quie, 1978). In children less than eight years of age, the incidence of otitis media was reported at 20%, and each successive episode increased the risk of further attack (Giebink and Quie, 1978). Brownlee (cited in Giebink and Quie, 1978) described the incidence of otitis media in 772 five year olds. Of this group, 84% were shown to exhibit at least one incidence of the disease. Recurrence was a major problem, 50% of these children had three or more episodes and 25% had at least six. According to his studies, the incidence peaked between the ages of 1 and 2 years and declined to <1% after the twelth year (Giebink and Quie, 1978). Brook (1979) studied middle ear effusions from 50 patients. They reported aerobic bacteria from 21 patients (42%) and anaerobic bacteria from 3 patients (6%). However, aerobic and anaerobic bacteria isolates growing together were recovered from 25 (50%) patients (Brook, 1979). Of the anaerobes isolated, three of the most common were Bacteroides fragilis (3), Propionibacterium acnes (3), and Peptostreptococcus sp . (3). Screening for anaerobic bacteria in middle ear aspirations, according to Brook (1980), showed them to be involved in over 50% of his cases. This and other recent satistics have agreed with the results published around the turn of the century which reported the recovery rate for anaerobes was 35-55% in patients with otitis media (Brook, 1980).

Response to antibiotic treatment by otitis media is quite variable

and many factors account for this. Antibiotic susceptibility of certain bacteria is affected by the quantity of bacteria within the middle ear (Wald et al. 1983). When colony counts are greater than 1×10^5 cfu/ml, the amount of antibiotic required to inhibit the growth of bacteria increases dramatically. Experimentally, Wald et al. (1983) found that persistance and reoccurence of chronic otitis media may be influenced by the initial concentration of bacteria in the middle ear fluid. In the same study, they showed clinically that 77% of all cases of otitis media showed an aerobic concentration of greater than 1×10^4 cfu/ml. Due to the requirement for accurate identification and quantitation of the bacteria causing the infection, an appropriate culture technique is necessary. Brook (1979) found that direct aspirations through the tympanic membrane were more reliable in establishing the bacteriology of chronic otitis media, which assisted in the selection of proper antimicrobial therapy.

Animals and Techniques used in Previous Studies

In the study of otitis media, guinea pigs and chinchillas have been the most common animals used for research. Fulghum et al. (1982) found that gerbils are an acceptable model for otitis media studies and, may survive infection better than chinchillas. The same bacterial infection that caused the deaths of four out of seven chinchillas caused the death of only three of twenty-three gerbils. Gerbils themselves have a low incidence of otitis media, but are susceptible to induced infection. Although most of the research has been performed on guinea pigs, the

reactions of guinea pigs can be associated with that of gerbils.

In artifical infection of guinea pigs, Thore et al. (1982) found that Propionibacterium acnes , a common component of skin flora, was most persistent in studies of anaerobic bacteria associated with otitis media. As a control they advocate sterilizing the external auditory canal prior to culturing. Their studies have shown that during infection a thickening of the mucosal lining occurred, and the bacteria eventually invaded the subepithelial space.

Gerbils, weighing between 50 and 100 grams, are currently being used by Fulghum and colleagues for a model of experimental otitis media. Since these animals can develop high serum cholesterol levels under specific diets, they are valuable in the study of cholesteatoma and its affects on anaerobic bacteria (Rich, 1968). Heterologous antiserum has also been prepared for the study of their immunological response.

Thore in 1982 has found that synergy plays an important part in extablishing infections in guinea pigs, and that when one bacterium is established it takes less of an initial concentration for a secondary infection to start. This is important since there is a possibility that the anaerobic bacteria of the upper and lower respiratory tract play a role in the establishment of chronic otitis media (Brook, 1979). His proposed explaination was that even with prophylactic treatment, many people show a recurrent infection.

Conclusion

Research has shown that anaerobic bacteria are found in up to 50% of

all cases of chronic otitis media. Because of this prevalence, research into this infection is important. Factors that affect the conditions inside the middle ear, such as cholesteatoma and inadequate eustachian tube drainage, establish environmental conditions that are favorable for anaerobic growth. Anaerobes have different antibiotic susceptibility patterns than aerobes, having been shown to exhibit increased resistence to aminoglycosides and penicillins. Standardized culture techniques are needed to obtain correct identification of the bacteria in order to prescribe the correct antibiotic. Gerbils have been shown to be an adequate animal model for the study of otitis media. With chronic otitis media producing many complications including, brain abscesses, hearing loss, balance dysfunction, and facial palsy, research is needed to accurately define the disease process. Knowledge into this process can lead to preventive treatment and cure of this infection.

PURPOSE OF THIS RESEARCH

In this research we will study the effects of different anaerobic bacteria on the gerbil middle ear. Standardized inoculum of single species of live anaerobic bacteria will be injected into the middle ear bulla of healthy animals. The resulting effects will be used as a baseline for further studies. Injections of comparable numbers of non-living bacteria of the same species will serve as control.

Demaria in 1984, has shown that bacteria were present in gram stained smears of 84% of middle ear effusions, however, 50% of them

showed no growth. Studies such as this suggested that an endotoxin was responsible for the pathogenesis of otitis media with effusion, and this endotoxin can cause the inflammatory cell response (Demaria, 1984). Other studies tend to limit the role of bacteria in serous otitis media. Brook, in 1983, in support of this stated that further studies were needed to evaluate the benefits of antimicrobial therapy in the pathogenesis of serous otitis media. Wong, in 1983, reported that an increase in polysegmented neutrophils indicated bacteria were likely involved in the infection. Also shown in the same study was that an increase in lymphocytes decreases the chance of bacteria being found in whatever effusion was present. These results implied that there were two types of otitis media, bacterial and non-bacterial. An increase in polymorphonuclear neutrophils would then indicate the infection is bacterial, and conversely if there were an increase in lymphocytes the infection would be nonbacterial. Another possible explanation implied is that bacteria were present to induce the initial inflammation and then rendered nonviable and an endotoxin perpetuated the inflammation. More research needs to be done to secure a true understanding into the etioloy of otiis media with effusion.

The research reported in this study will: 1) establish criteria for grading different degrees of infection within the middle ear using histological preparations; 2) assign a numerical grade of pathology to single innoculations of viable and non-viable bacteria to establish a baseline study; 3) assign a numerical grade of pathology to two organism mixtures of viable bacteria and compare these with the basline study to establish whether synergy was present; 4) assign a numerical grade of

pathology to three organism mixtures of living bacteria and compare these results with the baseline study and the two organism mixtures to establish if synergy is present.

TABLE I

MORTALITY RATES ACCORDING TO YEAR (BROOK, 1980)

Year	Mortality Rate
1939 and earlier	70.0%
1939-1949	40.0%
1950-1960	20.0%
1961-1971	10.0%

TABLE II

RESULTS OF TYMPANIOCENTESIS (BROOK, 1980)

Aerobic	
Staphylococcus pneumoniae	7.0%
Haemophilus influenzae	9.0%
Anaerobic	
Propionibacterium acnes	7.0%
Peptostreptococcus sp.	12.0%
Bacteroides fragilis	13.4%

TABLE III

INCIDENCE OF SPECIES IN THE STUDY (SUGITA ET AL, 1981)

Organism	Incidence
Peptococcus sp.	39.5%
Bacteroides sp.	22.0%
Peptostreptococcus sp.	7.4%

METHODS AND MATERIALS

ANIMALS Young adult Mongolian Gerbils (Meriones unguiculatus) of less than four months of age were used. These animals were obtained from Tumblebrook Farms and were acclimatized to the laboratory for at least two weeks before being used. Food and water were available for the animals as needed. Animals used in this study weighed between 50 and 70 grams. Before inoculation with bacterial suspensions, normal appearing acclimatized animals were anesthestized with 0.03 ml of a mixture of 10 ml Ketamine and 1.5 ml xylazine. These animals were then examined otoscopically for signs of infection, inflammation, excess ear wax, intact tympanic membrane, and any other signs of observable disease or abnormality. After anesthetization, the hair was removed from the exterior of the skull over the bulla and the skin disinfected with an iodine solution. The iodine solution was allowed to dry and inoculation preformed with a 26 gauge needle directly through the temporal bone into the bulla.

INNOCULATION PROCEDURES: Inocula were initially grown on prereduced, anaerobically steralized (PRAS) chopped meat (CM) agar as described by Holdeman, Cato, and Moore, 1977. These organisms were then transferred to peptone, yeast, glucose (PYG) liquid medium and were incubated from 12 to 24 hours or until visible growth appeared. The cultures were then diluted anaerobically with 5 ml of PYG medium to a concentration equal to that of one- half of a "Number One" McFarland standard (1.5×10^8 cfu/ml) and then 0.03 ml of this suspension was injected into each animal's bulla. For polymicrobic mixtures, after each of the organisms

were diluted to one-half of a "Number One" McFarland standard as above then 1 ml was transferred quantitatively into a single PRAS empty tube. From this mixture 0.03 ml was injected giving a uniform inoculation dose of 4.5×10^6 cfu. All incubations were at 37 degrees Centigrade and all transfers were carried out by strictly anaerobic methods (Holdeman, Cato, and Moore, 1977). Nonliving organisms were rendered non-viable by immersion in a water bath at 80 degrees celsius for 15-20 minutes. Aliquots of these cultures were then inoculated onto a roll tube and incubated anaerobically to establish if growth was present.

SAMPLING OF MIDDLE EARS: Animals were sacrificed at 3, 5, 7, 14, 21 and in some cases 28 days. Animals were anesthetized with 0.03 ml of the ketamine-xylazine solution, resulting in surgical anesthesia and allowing function of the cardiovascular system for adequate perfusion. Once anesthetized the animals were examined otoscopically using a surgical microscope for visual assessment of the infection. Animals are then surgically opened to expose the heart and a normal saline solution introduced into the aortic arch via needle insertion through the left ventricle. Once the infusion was started the right atrium was cut to allow drainage of the blood. After the saline has completely perfused the animal, approximately 10 minutes, a fixative of either Fischer Perfix R or 10% buffered formalin was used as a preservative. After the fixative has perfused the animal for approximately 3-5 minutes, the animals head was removed, skinned and placed in the fixative. Heads were then decalcified, dehydrated through a grades series of dilutions of alcohol to a final concentration of 100% alcohol. The heads were then

placed in tubules and allowed to saturate with xylene before embedding in paraffin. The heads, affixed in a paraffin block, were sectioned into 15 micron thick sections. Two slides were prepared of a coronal section through the head containing both right and left middle ears along with the tympanic membrane . One of the slides was stained with a routine hematoxylin and eosin (H and E) stain while the other was reserved for additional study.

MORPHOLOGICAL EXAMINATION OF THE SLIDES: Slides were examined via light microscopy for signs of histopathology and accumulations of white blood cells. These slides were then ranked on a scale of 1 to 5 with five being a fulminant infection. Details of grading scale will be discussed later in a separate section.

BACTERIA: Bacteria used in these experiments were either clinical isolates, obtained from active cases of otitis media, other pathogenic conditions, or cultures obtained from ATCC. The cultures used were:

Fusobacterium nucleatum ATCC # 10953

Escherichia coli ATCC # 11303-4

Peptococcus anaerobius ATCC # 27337

Propionibacterium acnes (Fulghum OM-4)

Clostridium perfringes CDC

Bacteriodes melaninogenicus assachrolyticus (Finegold 536)

Peptostreptococcus intermedius (Fulghum OM-2)

Gaffyka anaerobius (Finegold)

Peptococcus magnus (Finegold)

Bacteroides fragilis (Beamer)

Clinical isolates despite being identified in their respective laboratory were recharacterized in our lab following the methods described in the VPI anaerobe manual (Holdeman, Cato, and Moore). ATCC cultures were rehydrated and streaked on a roll tube for visual identification of colony morpholology. After identification of these organisms, samples were transferred to tubes of chopped-meat medium and incubated until visible growth was established. Then approximately 0.5 ml was transferrred to PYG media, grown to log phase and diluted to one half of a "Number One" McFarland standard prior to inoculation into middle ears of animals.

GRADING SCALE FOR THE ASSESMENT OF DAMAGE TO THE MIDDLE EAR FROM THE OBSERVATION OF SLIDES PREPARED FROM SECTIONS OF GERBIL SKULLS FROM ANIMALS WITH EXPERIMENTAL OTITIS MEDIA

The scale devised has been based on three objective categories: quantitation of white blood cells, the severity of bone damage/formation, and the degree of thickening of the periosteum. Each of these categories has been weighted according to estimated importance in its involvement in the course of damage. The categories are assigned a number according to their type, 0-8 for white blood cell's, 0-4 for bone damage/formation, 0-4 for thickening of the periosteum. A slide is viewed via light microscopy and evaluated numerically for each category. The category numbers are added to obtain a cumulative total ranging from 0-20. This range (0-20) is subdivided into 5 grades, 0-1=0, 2-4=1, 5-7=2, 8-10=3, 11-14=4, 15-20=5. The grading is such that 0 is the least severe or a normal ear and 5 is the most severe and thus has the most damage. A prejudice is included in this system so that any extensive bone damage with its resulting thickening in periosteum will give at its lowest a score of 4. In our experience, the gerbils that have shown most of the bone damage/formation, have also shown a increased presence of white blood cell's, thus giving most of these cases a grade of 5.

GRADE SCALE FOR THE CATEGORIZATION OF MIDDLE EAR INFLAMMATION

WHITE BLOOD CELL'S:

0= No white blood cell's.
1= One pocket present to localized, or present to localized in the middle ear.
2= One pocket with less than one-half of the total area filled, or two pockets with localized cell's.
3= One pocket with greater than one-half of the total area filled, two pockets with less than one-half of the total area filled, or three pockets with localized cell's.
4= Two pockets with greater than one-half of the total area filled, or three pockets with less than one-half of the total area filled.
5= Three pockets with greater than one-half of the total area filled but not overflowing into the middle ear.
6= Three pockets completly filled with overflow into the middle ear. Overflow is restricted to less than one-fourth of the total middle ear area.
7= Three pockets completly filled along with one-fourth to one-half of the middle ear.
8= Three pockets completly filled along with greater than one-half of the middle ear.

BONE:*

0= No damage.
1= Localized bone damage.
2= Bone damage to greater than one-tenth to less than one-half of the total perimeter of the bone.
3= Bone damage to greater than one-half of the total perimeter.

4= Bone damage to greater than one-half of the total perimeter with heavy new bone formation into the middle ear.

* the numbers obtained from this scale are to be multiplied by two.

PERIOSTEUM:

0= No thickening
1= Periosteum thickened to greater than or equal to three cell layers at less than one-fourth of the total perimeter.
2= Periosteum thickened to greater than or equal to three cell layers at less than one-half of the total perimeter, or greater than or equal to five cell layers at less than one-fourth of the total perimeter.
3= Periosteum thickened to greater than or equal to three cell layers at greater than one-half of the total perimeter, or greater than or equal to five cell layers at greater than one-fourth, but less than one-half of the total perimeter.
4= Periosteum thickened to greater than or equal to five cell layers at greater than one-half of the total perimeter.

GRADING SCALE

0-1=0 2-4=1 5-7=2 8-10=3 11-14=4 15-20=5

EXAMPLE:
Under light microscopy a slide is found to have three of the pockets filled with white blood cell's, but not overflowing into the bulla. A score of 5.

Bone damage/formation of greater than one-half of the total perimeter but not with heavy formation into the middle ear. A score of 3x2=6.

Periosteum is thickened to greater than 5 cell layers at less than one-half of the total perimeter. A score of 3.

The scores are added to obtain a raw ranking in this case 5+6+3=14. This value is expressed as a grade 4 infection.

Our grade 5 infection score is equivalent in histologic pathology to the infections experimentally induced by Fulghum(1982,1985,1985) in the gerbil with Streptococcus pneumoniae type 3, and Haemophilus influenza non-typable.

LEGEND FOR PHOTOGRAPHS

Plate 1A. Photomicrograph, from a coronal section of a gerbil head showing a normal bulla. This recieved a 0 ranking.

Plate 1B. This section recieved a ranking of 1 due to its low number of white blood cell's, normal periosteum, and lack of bone damage.

Plate 1C. A grade 2 infection showing localized white blood cell's and a slight thickening of the periosteum, and lack of bone damage.

Plate 2A. A grade 3 infection with accumulations of white blood cells in the "pocket" areas and thickening of the periosteum. Still no bone damage.

Plate 2B. A grade four infection showing large accumulations of white blood cell's and extensive thickening, of greater than 5 cell layers, of the periosteum.

Plate 2C. Nonlocalized bone damage, extensive white blood cell accumulations and extensive periosteum thickening elude to a grade 5 infection.

Plate 3A. A grade 5 infection showing moderate new bone formation.

Plate 3B. A grade 5 infection showing extensive new bone formation.

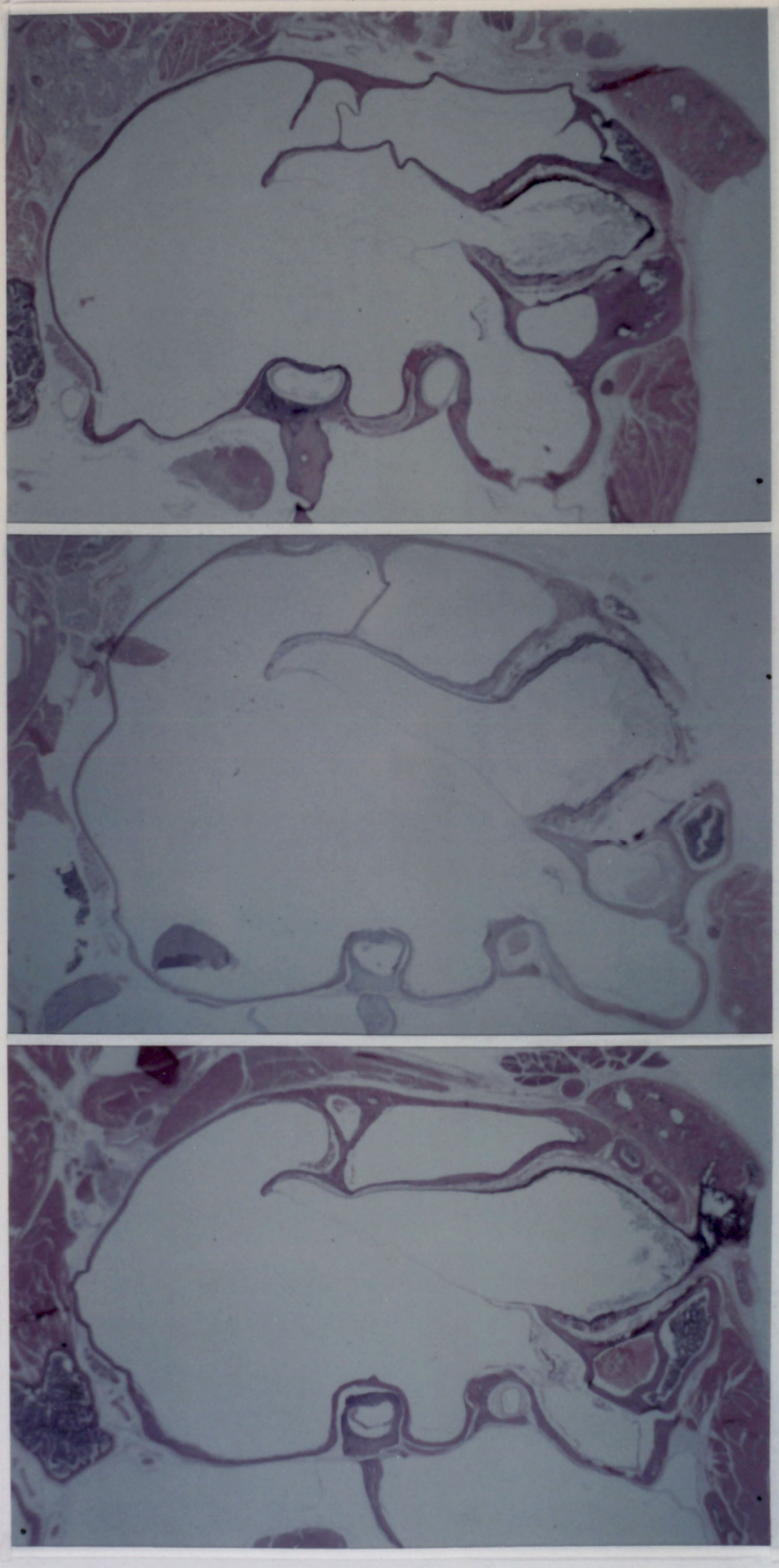
Plate 3C. A 200x photomicrograph of a gerbil middle ear, showing thickened periosteum integrated with white blood cell's and bone. Notice how the periosteum is pulled away from the bone. (200x)

Plate 4A. This plate shows the periosteum thickened with a layer of new bone deposited upon the old bone. (200x)

Plate 4B. New bone formation beginning to form into the middle ear. (200x)

Plate 4C. Section showing thickening of the periosteum to greater than or equal to five cell layers. (400x)

PLATE 1

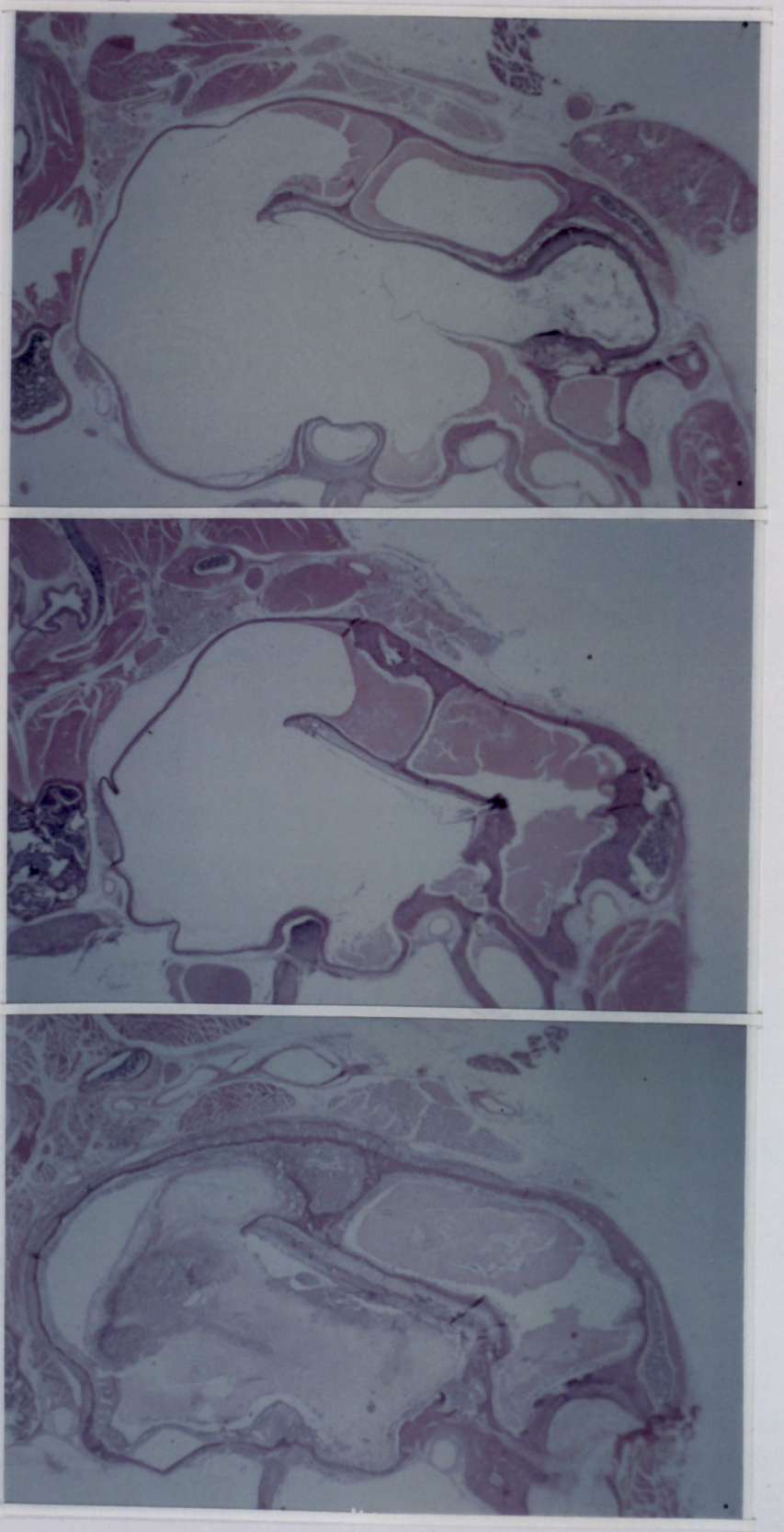


A

B

C

PLATE 2

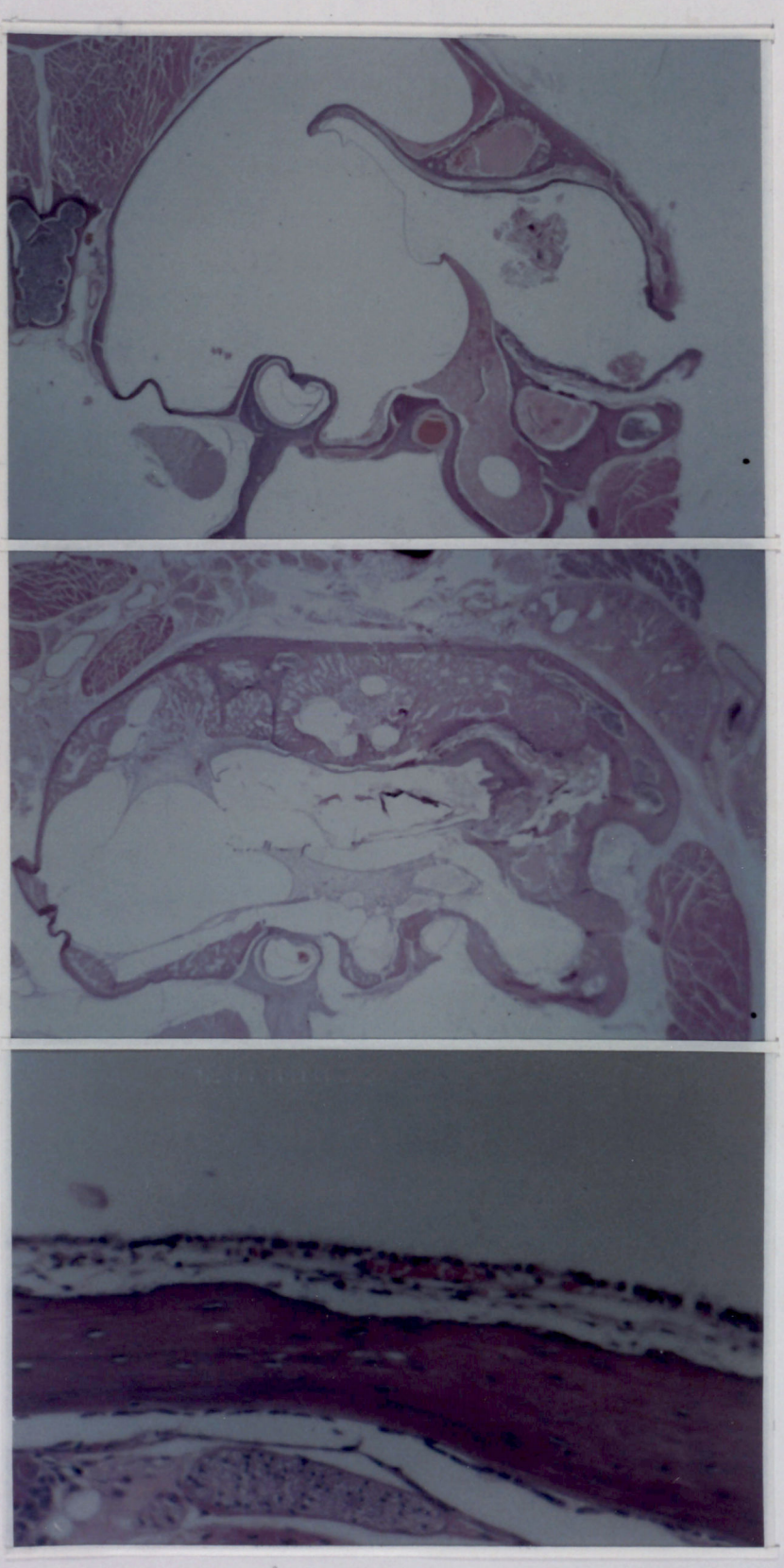


A

B

C

PLATE 3

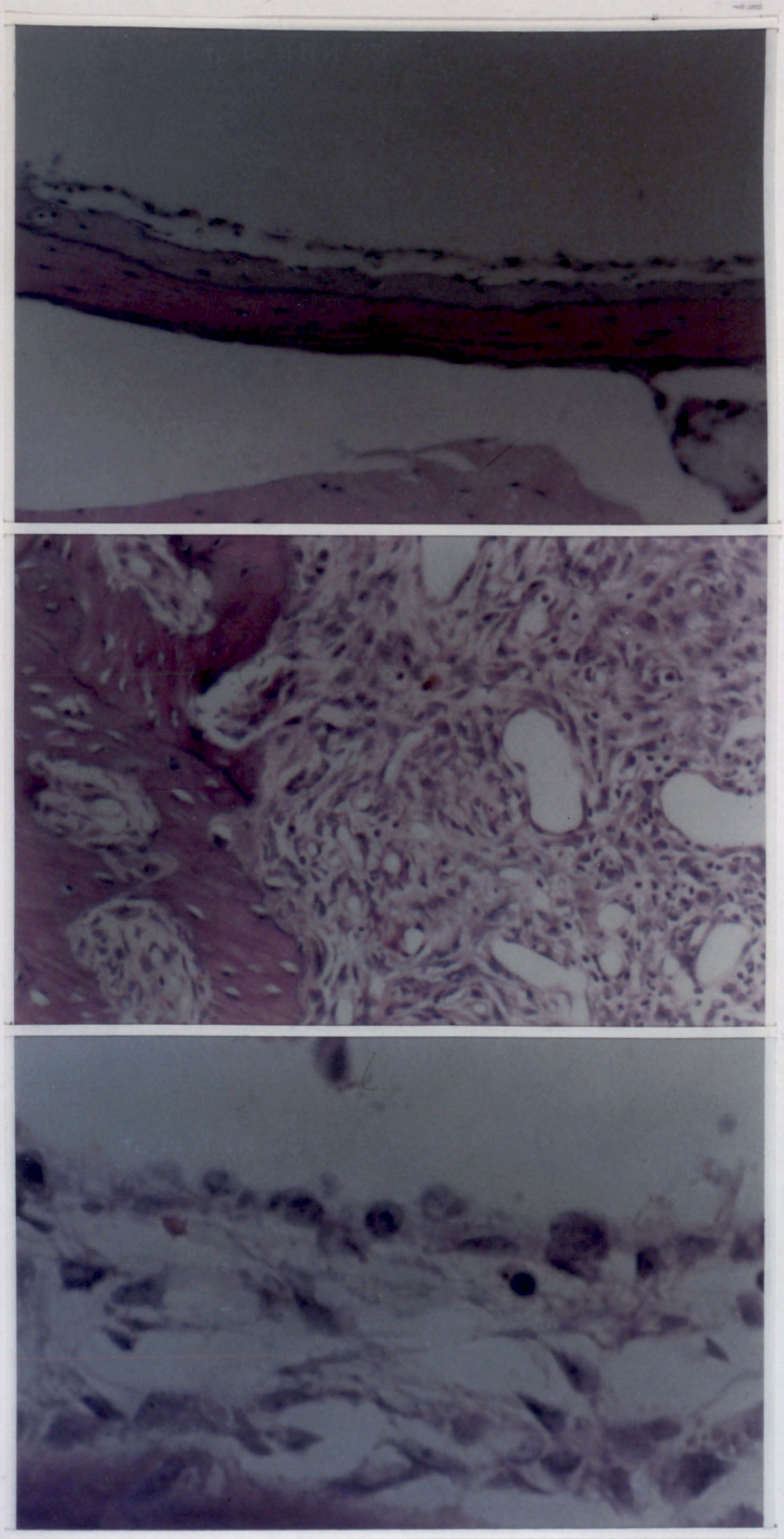


A

B

C

PLATE 4



A

B

C

TABLE IV

GRADE OF PATHOLOGY FROM SINGLE LIVING AND NON-LIVING INOCULATIONS

Organism	Living or Non-living	Sampling Period (Days) 3	5	7	14	21	28
Gaffyka anaerobius	Living	-	1	0	0	-	-
	Non-living	-	2	0	-	-	-
Peptostreptococcus anaerobius	Living	4	4	5	5	5	-
	Non-living	4	4	4	0	1	0
Bacteroides melaninogenicus	Living	3	4	1	5	-	-
	Non-living	2	1	1	0	0	-
Propionibacterium acnes	Living	2	1	2	0	-	-
	Non-living	2	4	4	5	-	-
Peptostreptococcus intermedius	Living	3	4	1	0	0	-
	Non-Living	3	5	1	0	0	-
Fusobacterium nucleatum	Living	4	2	3	0	2	3
	Non-living	2	4	2	0	0	0
Clostridium perfringens	Living	3	4	0	4	5	-
	Non-living	3	4	1	3	0	0
Bacteroides fragilis	Living	2	3	1	0	0	5
	Non-living	1	3	0	5	5	-
Peptococcus magnus	Living	1	2	1	-	-	-
	Non-living	1	1	0	0	-	-
Escherichia coli	Living	4	5	5	-	-	-
	Non-living	3	4	4	-	-	-

TABLE V

GRADE OF PATHOLOGY FOR DOUBLE LIVING INOCULATIONS

Organisms	Sampling Period (Days) 3	5	7	14	21
Escherichia coli x Bacteroides fragilis	-	4	5	-	-
Clostridium perfringens x Peptococcus magnus	4	4	5	4	3
Bacteroides melaninogenicus x Escherichia coli	5	4	5	5	-
Clostridium perfringens x Propionibacterium acnes	4	4	5	5	-
Fusobacterium nucleatum x Peptostreptococcus intermedius	3	3	0	0	-
Bacteroides fragilis x Fusobacterium nucleatum	3	3	2	0	-
Propionibacterium acnes x Peptostreptococcus intermedius	3	3	0	0	0
Bacteroides fragilis x Peptostreptococcus intermedius	2	3	0	5	4
Bacteroides melaninogenicus x Peptococcus magnus	4	3	0	0	0
Peptostreptococcus anaerobius x Peptococcus magnus	3	2	0	0	-

TABLE VI

GRADE OF PATHOLOGY FOR TRIPLE LIVING INOCULATIONS

Organisms	Sampling Period (Days) 3	5	7	14
Escherichia coli x Bacteroides fragilis x Peptostreptococcus intermedius	2	5	5	5
Bacteroides melaninogenicus x Peptococcus magnus x Peptostreptococcus anaerobius	3	3	4	5
Bacteroides melaninogenicus x Bacteroides fragilis x Propionibacterium acnes	3	5	2	0
Peptostreptococcus intermedius x Peptostreptococcus anaerobius x Bacteroides melaninogenicus	3	3	3	4
Peptostreptococcus intermedius x Propionibacterium acnes x Clostridium perfringens	2	1	5	0

RESULTS AND CONCLUSION

SINGLE LIVING AND NON-LIVING INOCULATIONS

In the single living and non-living groups inclusive, *Peptostreptococcus anaerobius* and *Escherichia coli* were the organisms that exhibited the greatest degree of pathology. Living *P. anaerobius* and *E. coli's* grade of pathology increased as days post inoculation increased. For non-living *P. anaerobius* the grade of infection decreased as days post infection increased while the results for non-living *E. coli* mimicked the living after day three. *Propionibacterium acnes* was the only organism whose grade of pathology was drastically increased in the animals inoculated with non-living cells compared to the living. This effect is different from most other bacteria used since it was expected that all non-living bacteria would exhibit a similar grade of pathology. Conversely, *Gaffyka anaerobius* , had the lowest grade of pathology for both the living and non-living inoculations, with all evidence of pathology absent by the last day of sacrifice.

DOUBLE LIVING INOCULATIONS

The double mixture organisms of *Bacteroides melaninogenicus* and *Escherichia coli* displayed the overall greatest degree of pathology of the organisms used. Synergy could be a factor since *Bacteroides melaninogenicus* did not attain this high of grade of pathology by itself

in the living phase. E. coli did achieve a high grade of pathology, but did not display the maximum on day three. Inhibition did not take place since the pathology resulting from inoculation of the mixture is equal to or higher in grade, than Escherichia coli and Bacteroides melaninogenicus used alone. C. perfringens and P. acnes displayed almost the same degree of pathology that the mixture of B. melaninogenicus and E. coli showed except on day 3 where B. melaninogenicus and E. coli displayed a higher grade of pathology. The mixture of Peptostreptococcus anaerobius and Peptococcus magnus displayed the least highest grade of pathology. It appeared that synergy does not take place since the single living inoculation of Peptostreptococcus anaerobius maintained a grade of two until day seven where the mixture decreases from two to zero on day seven. On day seven also Peptococcus magnus achieved a grade of one which was still higher than the zero achieved by the mixture. One can not say for sure whether inhibition took place, since the grade for the mixture compared closely with the grades for the single living inoculations.

Examples of double mixtures that were synergistic were Clostridium perfringens x Peptococcus magnus , Clostridium perfringens x Propionibacterium acnes first week only, and Bacteroides fragilis x Peptostreptococcus intermedius second week only. Examples of double mixtures that were definitely inhibitory were Peptostreptococcus anaerobius x Peptococcus magnus second week only, Fusobacterium nucleatum x Peptostreptococcus intermedius , Propionibacterium acnes x Peptostreptococcus intermedius , and Bacteroides fragilis x Peptostreptococcus intermedius only in first week. Most of these results

were based on a visual examination of the data. However, statistical analysis was performed on the double mixtures exhibiting the highest and lowest grade of pathology for the first week. This analysis used the Kruskal-Wallis test and determined there was no difference between the single organisms used for the highest grade. There was also no difference between the single organisms used for the lowest grade either.

TRIPLE LIVING INOCULATIONS

The triple mixture of Escherichia coli , Bacteroides fragilis and Peptostreptococcus intermedius achieved the highest grade of pathology of all of the triple mixtures. It is debatable whether synergy has taken place in this mixture. Bacteroides fragilis and Peptostreptococcus intermedius in the single living inoculations only achieve low grades of infection that on their highest grade were only at that peak for one day, while the mixtures attained a grade of five on day five which lasted through day fourteen. Escherichia coli used alone however, achieved high grades of infection throughout the study period, therefore we cannot assertain whether the high grade of infection in the mixture was due to synergy or Escherichia coli alone. We can say however, that inhibition did not take part since such a high grade of infection was found. The lowest grade of infection for the triple mixtures was found in Peptostreptococcus intermedius , Peptostreptococcus anaerobius , and Bacteroides melaninogenicus where it maintained a steady grade of three on days three, five, and seven and increased to four on day fourteen. Synergy probably plays a part in this mixture since none of the single mixtures achieved a high grade of infection for greater than one day.

Inhibition also might play a part in the infection process since the mixture grade of infection never achieved the peaks that occurred with use of some of the individual cultures. What one sees is a leveling affect causing neither fulminant infection or amelioration of the degree of pathology.

Examples of triple mixtures that were synergistic were Bacteroides melaninogenicus x Bacteroides fragilis x Propionibacterium acnes during the first week only. Organisms that were definitely inhibitory were Bacteroides melaninogenicus x Bacteroides fragilis x Propionibacterium acnes second week only. Most of these results were based on a visual examination of the data. However, statistical analysis was performed on the first week data of the highest and lowest grade of payhology of the triple mixtures. This analysis used the Kruskal-Wallis test to determine if there was a statistical difference between the single organisms and the triple mixture. The results of this test showed no difference between the organism's exhibiting the highest grade of pathology and it's single organisms as well as the organism's exhibiting the lowest grade of pathology and it's single organisms.

CONCLUSION

Individual species of anaerobic bacteria are shown to have different effects when artificialy introduced into the middle ear. Some species of bacteria produce an acute infection that includes a rapid accumulation of white blood cells, mucous, and thickening of the periosteum. Others produce more chronic effects, and lead to excessive bone damage along

with the typical leukmoid reaction. With most species the pathology occurred reguardless of wheather the culture inoculated was living or non-living. The effect observed for the individual species can be different when mixtures of two or more species are injected into the middle ear. Some mixtures produced pathological synergy increasing the grade of infection, while others appeared to repress the grade of infection to less than what was produced by a single species. Results from the majority of the inoculations were inconclusive since it was difficult to determine whether any inhibition or synergy occurred. It is worthy to note that most of the inoculations that had high day three readings dropped one or two points on the next sacrifice before increasing again on the third sacrifice. First week data from the single living and non-living was used for statistical analysis. The Mann-Whitney test, was used to determine if there was a statistical difference between gram negative and gram positive bacteria, this test showed no difference between these organisms. A difference was noted between the gram positive living and non-living organisms according to the Kruskall-Wallis test. No difference was noted between the gram negative living and non-living organisms. These results support the theory that an endotoxin plays a significant role in the pathology of gram negative non-living infections.

These experiments were performed to establish methods and a data base for future research. In this study, our results were compared to those of Fulghum(1982,1985,1985). Our grade 5 infections were comparable to the experimental infections of Haemophilus influenza type b and Streptococcus pneumoniae type 23 in Fulghums work. Conditions and

organisms have been identified that requires additional investigation to more precisely define the inhibition or enhancement of the disease process. Since only one experimental animal was used for each sacrifice time, no correction could be made for their individual differences in susceptability. In future experiments more than one experimental animal should be sacrificed after each established period, and replicate data collected. At least four animals should be sacrificed after each period and the periods of sampling should be less than three days days apart.

Manifestations of these experiments are extremly broad. Antimicrobial testing should yeild valuable data of its effects on organisms within the middle ear. Cellular components of various bacteria injected into the middle ear would also produce useful information on the role of non-living bacteria in this disease. Tympanocentesis performed on all animals, and the micro-organisms isolated and identified would establish which organisms or mixtures of organisms exhibit synergy or inhibitions. Many physical manifestations can be based on our grading scale. High frequency sound could be used to establish what degree of damage within the middle ear caused hearing loss.

The results of such studies should enable the etiology of Otitis media to be further elucidated and this may point the way to the benefit of adequately treating this serious and crippling disease.

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