Experimental Otitis Media in Gerbils and Chinchillas with
Streptococcus pneumoniae, Haemophilus influenzae, and
Other Aerobic and Anaerobic Bacteria

ROBERT S. FULGHUM,1* JACK E. BRINN,2 A. MASON SMITH,1 HAL J. DANIEL III,3 AND
PATRICIA J. LOESCHE3

Department of Microbiology1 and Department of Anatomy2, School of Medicine; and Department of Speech,
Language and Auditory Pathology, School of Allied Health,3 East Carolina University, Greenville, North
Carolina 27834

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To ascertain the usefulness of Mongolian gerbils as an inbred model for otitis media, 52 Mongolian gerbils (Meriones unguiculatus, strain MON/Tum) were compared with 26 chinchillas (Chinchilla laniger) for susceptibility to Streptococcus pneumoniae type 3, Haemophilus influenzae type b, and a polymicrobial culture including anaerobes (Streptococcus intermedius, Propionibacterium acnes, Staphylococcus epidermidis, and Corynebacterium sp.). Organisms were inoculated percutaneously into the superior chamber of the middle ear bulla. The gerbils and chinchillas shared similar susceptibilities and responses to the inoculated organisms as determined by X-ray, otoscopic, histopathological, and microbiological determinations at 5 to 7 days. Koch’s postulate studies proved the role of S. pneumoniae and H. influenzae in the pathology found in both animal models. The animals were also susceptible to the polymicrobial culture, although the relative virulence of the individual members of this mixture was low, suggesting that these species potentiated as a polymicrobial mixture. The Corynebacterium sp. appeared to elicit the greatest histopathological response in chronic (8-week) studies in gerbils. The gerbils were found to be useful as an alternative animal model for the study of otitis media of bacterial etiology.

Despite the use of appropriate antibiotic therapy against the most common bacterial etiological agents of otitis media, children are often predisposed to recurrent attacks of otitis media (5, 7, 13, 23, 24, 28). It is now generally accepted that Streptococcus pneumoniae and Haemophilus influenzae are, in that order, the most important etiological agents in more than one-half of the cases of otitis media, whereas Staphylococcus aureus, Branhamella catarrhalis, coagulase-negative staphylococci, and enteric bacilli are infrequent causes (11, 19, 20, 30). Phagocytic cell types found in otitis media with effusions now point to a microbial role in its etiology (22). Many studies show no significant pathogen or they find “sterile” specimens (some due to inappropriate culture methodologies?) from a significant proportion of all cases of otitis media (e.g., 7, 17, 19). Polymicrobial infection flora (many including anaerobic bacteria) are found in some others, especially in recurrent acute and in chronic otitis media (1, 2, 6, 18, 33). The role of other isolates from cases of otitis media is uncertain, the etiology of otitis media remains incompletely defined (13, 27), and recent symposia on otitis media call for further study of the etiology of the disease (27).

It has been shown that the chinchilla is a useful model for studies of pneumococcal otitis media (8–10, 12, 21). The lack of inbred strains of chinchillas and other factors reduce its suitability; therefore, alternative animal models for otitis media research are needed (27). One animal that will serve is the Mongolian gerbil, Meriones unguiculatus (4, 31), which is available as inbred strains. The anatomy and histology of the eustachian tube and middle ear cavity of these gerbils are quite similar to those of chinchillas (3a).

The gerbil meets the criteria of Lewis et al. (21) for an acceptable animal for a model for otitic infection. We have studied the indigenous microflora of the nasopharynx and middle ear of gerbils and find them to be microbiologically acceptable as model animals for otitis media studies (34).

The main purpose of this investigation was to determine the susceptibility of Mongolian gerbils to otitis media when inoculated with Streptococcus pneumoniae type 3, H. influenzae type
b, and the anaerobic polymicrobial otitic infection consisting of Propionibacterium acnes, Streptococcus (Peptostreptococcus) intermedius, Staphylococcus epidermidis, and a Corynebacterium species (unidentifiable) (6). Results of inoculations were evaluated by behavioral, clinical, and histopathological studies.

We found the gerbils to be susceptible to induced otitis media due to S. pneumoniae type 3 and to H. influenzae type b. Some variability was found in inducing otitis media with the other bacterial species in pure culture; however, the gerbils were susceptible to the entire polymicrobial infection, including the anaerobic bacteria, and to the Corynebacterium sp. Both the chinchillas and the gerbils share similar susceptibilities to otitis media induced by the organisms used in this study.

**MATERIALS AND METHODS**

**Animals.** Healthy young adult (4 months of age) Mongolian gerbils (M. unguiculatus, inbred strain MON/Tum) were maintained as suggested by Schwentker (31). One week before use, each gerbil was anesthetized with a mixture of 87 mg of ketamine and 13 mg of xylazine per kg (35) and examined otoscopically for signs of otitis media, for intact tympanic membrane, and for any other observable disease or abnormality. Gerbils whose external auditory canal was too small to accommodate a 2-mm-diameter stainless-steel otological speculum were surgically prepared by making a dorsal-ventral incision in the external auditory meatus and pinna anterior to the tragus, cutting through the cartilage and to the temporal bone auricular orifice (G. Scott Giebink, personal communication). In some gerbils, the entire tragus was excised. Wounds were washed with povodine-iodine (Betadine) surgical scrub. Animals were allowed to heal for 1 week before further examination and use. Animals prepared in this manner suffer no ill effects. This procedure allows study of almost all of the tympanic membrane when examined carefully with an otoscope or operating microscope.

**Bacterial cultures.** S. pneumoniae ATCC 6303 was obtained from the American Type Culture Collection and passed through mice to enhance pathogenicity. H. influenzae type b was isolated from a child with an eye infection by P. Bruce Campbell and was characterized in his laboratory.

The component cultures of the polymicrobial mixture 1 were isolated during our previous study of children with recurrent otitis media (6). The P. acnes and S. (P.) intermedius isolates were characterized by the criteria and methodology of Holdeman et al. (16).

The S. epidermidis isolate from the previous study (6) was later recharacterized (D. Colwell, W. Allen, and R. Fulghum, manuscript in preparation).

The Corynebacterium sp. isolate was also from our previous study (6). Cecil Cummins (personal communication) has determined the organism to be of the lipotrophic coryneform group of Smith (32), the species of which are not identified. Our strain grows slowly on tellurite agar, producing dark gray colonies after prolonged incubation (M. S. Strickland, personal communication). It was found not to be a Mycobacterium and not closely related to the coryneform JK group being studied by Robert E. Weaver (personal communication).

**Inoculation suspensions and procedure.** Inocula were prepared from growth in the liquid portion of prerduced, anaerobically sterilized chopped meat medium (16) for the anaerobes and from growth on Todd-Hewitt agar or Trypticase soy agar (BBL Microbiology Systems) containing 5% sheep blood for the aerobic organisms. Cell suspensions were made in Todd-Hewitt broth from agar media just before use. Inocula involving mixtures of organisms were made by mixing equal parts of each suspension of organisms. Suspensions of approximately 106 organisms/ml were used except for S. pneumoniae, which was 104 organisms/ml. H. influenzae inocula were approximately 105 organisms/ml. Inoculation procedures were similar to those used by Giebink et al. for chinchillas (12). Right ear bullae were inoculated, the left serving as controls.

**Incubation in inoculated animals.** Animals were held for varying incubation times, usually 1 to 7 days. Some of the animals were held for 8 weeks. Animals were examined two or three times per week for signs of otitis media. Animals held for more than 2 weeks were examined weekly.

**Behavioral examination.** At each examination, animals were observed for external signs of otitic infection. These include inability to maintain an upright stance, cantaing of the head, running in circles, twisting (circuiting) in one direction when held upside down by the tail, and signs of acute infection such as ruffled fur, lethargy, etc.

**Otoscopic examination.** After anesthesia with ketamine-xylazine, each animal was examined otoscopically for signs of otitis media, including fluid behind the tympanic membrane.

**Radiopacity examination.** Anesthetized animals were placed on a film cassette and irradiated with 150 kV and 15 mA for 0.2 s at a distance of 40 inches (ca. 100 cm). Animals having tissue swelling and infiltration of leukocytes or, in chronic responses, thickening of the mucoperistium and new bone deposition show radiopacity in the middle ear cavity (14, 15).

**Specimens collected.** When present, middle ear fluids were collected by tympanocentesis. One drop was plated on a culture medium, and the remainder was saved for future immunological studies. Cardiac puncture was used to obtain blood for the immunological studies. Bile, when present, was also aspirated from the gall bladder for the immunological studies.

**Histological preparations.** Each animal was fixed for histological studies by per cardiac perfusion and then decalcified, embedded, sectioned, and stained by our previously described methods (3a). Coronal sections 8 µm thick were made from three areas (the ventral anterior bulla area near the tympanic osteum, the inner ear-tympanic membrane area, and the dorsal bulla or superior posterior chamber area) and were stained with hematoxylin and eosin. Selected additional sections were stained with Gram stain.

**Koch's postulates studies.** Specimens from animals with an induced infection showing classic signs of otitis media were plated on appropriate media as described above, isolated, and partially recharacterized. Isolates belonging to the same species used to induce the initial case were then inoculated into
healthy animals. Koch's postulates were considered fulfilled if the second series of inoculated animals showed the same signs of otitis media as the first and if the etiological agent could again be isolated from these animals.

RESULTS

Effect of media. To determine the effect of injecting fresh, sterile, fluid bacteriological medium into the middle ear cavities of gerbils and chinchillas, approximately 0.2 ml of medium was injected into the middle ear bulla. In some cases small amounts of the media could be detected behind the tympanic membrane by otoscopic observation. Other than this, neither lasting effect nor gross pathology was observed, and the fluid medium cleared within 2 to 3 days. Histopathological evaluation showed no histological changes from normal.

Behavioral signs. Behavioral signs were found to be of limited use in determining the induction of otitis media in our animals. Where found, they were useful to denote that the animals were infected, but were not useful in predicting incidence of infection.

Radiopacity observations. Figure 1A shows a chinchilla with full radiopacity 7 days after infection with S. pneumoniae; Fig. 1B is that of a gerbil with a 7-day infection with mixture 1. Similar results were seen in gerbils and chinchillas with infections due to the other organisms. In general, radiopacity is a useful sign; however, since dead animals almost never showed the radiopacity even though fluid and organisms were found in the middle ear cavity, the radiopacity was due to swollen tissue, massive infiltration of phagocytic cells, or new bone deposition. Greselin (14) found infusions and thin pus not to be opaque.

Otoscopic observations. Inspection of the tympanic membrane with an otoscope or operating microscope was a very useful procedure in determining the presence of infection. The normal tympanic membrane in gerbils is slightly whitish-pink and translucent. In infected animals, the membrane would range from signs of mild inflammation to gray to a dark brownish-yellow opaque color with a very rough surface texture. Some showed whitish opaque areas during the infection, whereas in still others pus or fluid could be seen behind the tympanic membrane. In general, the progression of signs of inflammation to opaque tympanic membrane to a dark, rough-appearing membrane was seen with progression from shorter to longer times after injection of organisms, especially with mixture 1, which was studied over an 8-week period. In a few animals after injection, tympanic membranes would appear normal, yet histopathological evaluation showed results of a mild infection. It is unfortunate that the Karl Storz-Hopkins 2.7-mm 0° telescope will not fit into the external auditory canal of the gerbil for photographing the gerbil tympanic membrane (3).

Koch's postulates studies. Koch's postulates were considered to be fulfilled when S. pneumoniae and H. influenzae were used, proving that each organism used to induce the disease was indeed the pathogen responsible for the clinical signs and histopathology. The counts per

![Roentgenogram of chinchilla skull showing radiopacity in the right middle ear cavity (X-ray film positioned so that right ear is to your right) 7 days after inoculation with S. pneumoniae. (B) Gerbil with 7-day infection with mixture 1.](http://iai.asm.org/Downloaded-from/http://iai.asm.org/)

FIG. 1. (A) Roentgenogram of chinchilla skull showing radiopacity in the right middle ear cavity (X-ray film positioned so that right ear is to your right) 7 days after inoculation with S. pneumoniae. (B) Gerbil with 7-day infection with mixture 1.
milliliter of organisms aspirated from middle ear fluid were greater than counts per milliliter of inoculum.

**Spread of organisms from inoculated ear and death.** *S. pneumoniae* caused death in 4 of 7 inoculated chinchillas and in 3 of 23 inoculated gerbils. *H. influenzae* did not kill any gerbils, even though it developed clinical signs of infection in all 14 inoculated. It did, however, kill one of the three chinchillas inoculated, and each of the surviving chinchillas showed clinical signs of infection. Thus, although the gerbils were susceptible to both *S. pneumoniae* and *H. influenzae*, they were less likely to die from these infections. *H. influenzae* was observed to spread from the right middle ear to the left middle ear, presumably via the eustachian tubes, in two of seven gerbils studied for histopathology and to the eyes in another.

**Histopathological observations.** Figures 2 and 3 show representative histopathology induced by inoculating the organisms listed in Table 1. The histopathology of *S. pneumoniae* type 3 in gerbils 5 days after inoculation revealed elevation and vascularization of the mucoperiosteum with infiltration into the middle ear cavity of a large number of polymorphonuclear leukocytes (PMN). No evidence of spread of the pneumococci from the right (inoculated) middle ear cavity to the left (uninoculated) middle ear was

FIG. 2. Effect of inoculation of *Corynebacterium* sp. JOM138 into the middle ear cavities of gerbils: (A) At 5 days; (B) At 8 weeks; (C) At 8 weeks. Magnification 80×; hematoxylin and eosin stain.
The appearance of the tympanic membrane by otoscopic observation was that of an inflamed and ruptured membrane with pus in the external auditory canal at 2 days. At 5 days the tympanic membrane had become opaque and white. Although only two specimens were so tested, we found no evidence of invasion of the circulatory system by pneumococci.

The pathology of the middle ear of gerbils due to *H. influenzae* inoculation and 5 days of incubation was quite similar to that produced by *S. pneumoniae* except that the infiltrate also contained some monocytes and macrophages. Otoscopic observations of *H. influenzae*-inoculated animals showed inflamed tympanic membranes, often with fluid behind them. At about 2 weeks after inoculation the membranes had taken on a whitish opaque appearance.

Figure 2A shows the effect after 5 days of inoculating the *Corynebacterium* sp. into gerbils. In these animals, histopathological findings included a bloody exudate containing PMN and a thickened mucoperiosteum. At 5 days after inoculation with the *Corynebacterium* sp., the
tymanic membrane was inflamed and also had a grayish, granular appearance. In one animal into which this Corynebacterium was injected 8 weeks before sacrifice, striking changes were seen. Large growths of periostium intruded into the middle ear cavity appearing as a mass of loose connective tissue with fibroblasts, lymphocytes, and plasma cells. Osteoblasts were also seen, as was a remarkable amount of new bone formation. Some PMN and eosinophils were seen on the surface of these growths in the middle ear cavity (Fig. 2B, C). The left ear of this gerbil was entirely normal.

The S. intermedius-inoculated gerbils at 5 days showed a slightly elevated mucoperios- tum, evidence of a bloody exude containing eosinophils and lymphocytes as well as PMN. The left ear was entirely normal. Otoscopic examination demonstrated an inflamed tympanic membrane, a pronounced yellowing of the malleus, and evidence of fluid. Otoscopic and X-ray observations were normal by the 10th day. Results with S. epidermidis were similar except that the PMN response to inoculation was strikingly weaker. At 8 weeks both ears were entirely normal by all modes of evaluation.

P. acnes-inoculated gerbils showed a weak radiopacity in two of three animals observed. The tympanic membrane was slightly inflamed at 5 days and contained patchy areas of opacity. The bones of the malleus appeared to have become more yellowish. Histopathology revealed a slight thickening of the mucoperiosteum and a few PMN at 2 days, with little evidence of pathology seen in 5-day and 8-week animals.

When the individual species of mixture 1 (the Corynebacterium sp., S. intermedius, S. epidermidis, and P. acnes) were recombined and inoculated into gerbils and chinchillas, infections exhibiting signs similar to those produced by S. pneumoniae and H. influenzae were produced. Figure 3A shows a histological section of the right middle ear of a chinchilla which had been inoculated with mixture 1 in the right but not the left middle ear. After 7 days incubation, a swollen and thickened mucoperiosteum with many PMN in the exudate in the middle ear cavity was seen. Gram-stained slides of adjacent sections (Fig. 3B) revealed many bacteria in the exudate and PMN. Similar findings were seen in gerbils. Figure 3C shows the periostium of a gerbil 8 weeks after inoculation with mixture 1. Large ingrowths of periostium were seen in this animal as well as new bone overlying existing bone. Otoscopic findings progressed from an inflated bulging tympanic membrane to a dark, rough-appearing membrane.

**DISCUSSION**

Gerbils are known to have a low incidence of otitis media when reared under laboratory conditions (4, 26). Although anatomy, maintenance of an upright stance for prolonged periods (P. J. Loesche, M.S. thesis, East Carolina University, Greenville, N.C., 1975), and other factors con-
tribute to this natural resistance to otitis media, the nasopharyngeal bacterial flora may additionally act as a barrier to the carriage and establishment of the common etiological agents of the disease (34). Direct evidence for this is lacking, however; we did not find the common etiological agents of otitis media in the indigenous nasopharyngeal flora of gerbils (34), nor have Giebink et al. (10) found \textit{S. pneumoniae} in the indigenous nasopharyngeal flora of chinchillas. Giebink et al. found that colonization with \textit{S. pneumoniae} in chinchillas required the internasal inoculation of $10^4$ or more viable \textit{S. pneumoniae} cells (9).

Therefore, we suggest that a low natural incidence of otitis media and innate susceptibility to an experimentally induced etiological agent are two separate phenomena.

The use of moderately sized inocula, the development of signs and symptoms, the histopathology, and the fulfillment of Koch's postulates all point to the susceptibility of Mongolian gerbils to infections caused by the \textit{S. pneumoniae} and \textit{H. influenzae} strains used in this study. We judge gerbils to be as susceptible to, but probably less likely to die from, these experimental infections as chinchillas. We therefore propose the Mongolian gerbil as a model useful in studying middle ear infections for the following reasons: (i) these gerbils are available as inbred strains; (ii) they are readily available from commercial laboratory animal breeding corporations at reasonable prices, (iii) they are anatomically and microbiologically acceptable as a model system; and (iv) they are appropriately susceptible to the major etiological agents of otitis media.

Of major importance in the development of an acceptable microbiological model is the characterization of basic immunological parameters associated with the animal. A search of existing literature revealed that little was known about either the humoral or cell-mediated immune responses in gerbils. Studies have been undertaken using several traditional particulate and soluble antigens, and the immune responses of the gerbils have been investigated. After immunization, we were able to detect strong humoral responses to \textit{S. pneumoniae} type 3 and \textit{H. influenzae} type b strains used in this initial study. More important, we have prepared heterologous antisera to gerbil immunoglobulins which have provided valuable probes with which to study humoral as well as localized immune responses to bacteria, using enzyme-linked immunosassays. Results of these studies will be reported in a subsequent paper.

In regard to the other bacteria and polymicrobial infection flora used in this study, much more remains to be done to show clear results. For example, mixture 1 and the \textit{Corynebacterium sp. JOM138} appear to cause observable signs and striking histopathology in gerbils at 8 weeks of incubation. However, the requirement for infection of a large inoculum in medium, as well as the fact that washed bacterial inocula are rapidly cleared, suggests that these organisms would probably be able to act pathogenically only as secondary or opportunistic invaders in, for example, otitis media with effusions where conditions are more suitable than in a healthy middle ear. The same observation applies to the pure culture inocula of \textit{S. intermedius}, and \textit{P. acnes}. Therefore, we feel the use of inoculum in a medium simulates the compromised middle ear and is an intermediate step in the progression toward recurrency or chronicity. The injection of fresh, sterile medium alone has been found to produce no discernible effect in gerbils. The theory that we have proposed needs much further work and may be studied in something akin to the Paparella model of serous otitis media prepared by surgical occlusion of the eustachian tubes.

Synergy of weak pathogens in mixed infections is well known (25, 29). Weak pathogens, each having only a partial armamentarium of pathogenic factors, and bacteria serving to support these partially pathogenic bacterial species may, in the proper mixture and in suitable conditions, simulate the effect of much more highly pathogenic species which are pathogens in pure culture. Thus, mixtures of species may interact among themselves and with the host to produce pathology. In chronic otitis media the mixture may not always have to be the same combination or proportions of organisms, and this may be dependent not only on the pathogenic and support characteristics of any given mixture but also on the stage of the infectious process and the condition of the host. The gerbil model should be useful in studying the natural progression from acute infection to the polymicrobial flora of chronicity. Understanding the events and parameters surrounding this phenomenon should lead to improved modes of treatment and prevention. The use of the inbred strain will also facilitate further in-depth studies of the biology of the response to otitis media, thereby opening the way to better methods of vaccine development and evaluation as well as improved modes of treatment for both acute and chronic forms of bacteria-induced otitis media in humans.

\textit{S. epidermidis} has been implicated as a pathogen in some human cases of otitis media (5). In this study it elicited only a very mild, transitory pathology which caused no permanent change in the middle ear of our experimental animals. Gerbils and chinchillas have a predominantly \textit{Staphylococcus} indigenous flora in the naso-
pharynx (34), and therefore these animals may be unsuitable for pure culture studies of *S. epidermidis* and other coagulase-negative staphylococci.

In conclusion, we have presented inbred Mongolian gerbils as appropriately susceptible animals for the laboratory study of otitis media, its sequelae, and its treatment and prevention, since gerbils are susceptible to induced otitis media due to *H. influenzae* type b, to *S. pneumoniae* type 3, and to our mixture 1. Gerbils may also serve as models for otitis media induced by other microorganisms such as *Corynebacterium* sp. and other polymicrobial infections, including anaerobic bacteria. Otoscopic findings of inflammation and other changes in the tympanic membrane and histological findings, including infiltration of phagocytes, elevation of the mucoperiosteum (often with increased vascularity and sometimes with increased layers of cells) and, in some animals, formation of new bone, indicate an inflammatory response to the organisms used in this study. Permanent changes were seen in some of the animals held for chronic studies. With minor variations, chinchillas and gerbils appear to share similar susceptibilities to otitis media induced by the organisms used in this study.

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LITERATURE CITED