INFECTION AND IMMUNITY, May 1994, p. 1710–1718
0019-9567/94/$04.00+0

Vol. 62, No. 5

Synergistic Effect of Adenovirus Type 1 and Nontypeable *Haemophilus influenzae* in a Chinchilla Model of Experimental Otitis Media

KENJI SUZUKI† AND LAUREN O. BAKALETZ*

Otological Research Laboratories, Department of Otolaryngology, The Ohio State University College of Medicine, Columbus, Ohio 43210

Received 21 December 1993/Accepted 9 February 1994

We recently reported the development of a chinchilla model of experimental otitis media (OM) that uses a pediatric clinical isolate of adenovirus type 1 (4) and in which an active infection with the wild-type strain was demonstrated. To expand upon these findings, this study was designed to determine whether we could demonstrate adenovirus infection-induced predisposition to bacterial OM in the chinchilla, as has been shown in human epidemiological studies (D. A. Clements, F. W. Henderson, and E. C. Neebe, p. 27–29, *in* D. J. Lim, C. D. Bluestone, J. O. Klein, D. J. Nelson, and P. L. Ogra, ed., *Proceedings of the Fifth International Symposium on Recent Advances in Otitis Media*, 1993; F. W. Henderson, A. M. Collier, M. A. Sanyai, et al., N. Engl. J. Med. 306:1377–1383, 1982). In addition, we were interested in determining whether altering the order of pathogen acquisition would further affect the outcome of disease incidence and severity. Toward this end, cohorts of chinchillas were inoculated intranasally with a strain of nontypeable *Haemophilus influenzae* (NTHi) (86-028NP) which colonizes the chinchilla nasopharynx but does not consistently induce culture-positive OM when inoculated intranasally (L. O. Bakaletz, T. M. Hoepf, D. J. Lim, and B. Tallan, Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990, abstr. B-66, p. 37, 1990), adenovirus type 1 and then inoculated 7 days later with NTHi, NTHi and then inoculated 7 days later with adenovirus type 1, or both pathogens concurrently. All cohorts were observed over a 35-day period and assessed for incidence and severity of OM by several methodologies. The data collectively indicated that all animals receiving both pathogens developed OM of greater severity than those receiving only a single agent. Adenovirus inoculation followed 7 days later by NTHi inoculation was the order of pathogen acquisition which induced the most prolonged presence of NTHi in both the nasopharynx and the middle ear, the most severe tympanic membrane inflammation overall, and the most significant damage to and altered function of both middle ear and eustachian tube mucosae.

Epidemiological evidence has long supported the association of antecedent or concurrent viral upper respiratory tract infection with the development of bacterial otitis media (OM), and several viruses, including adenovirus, influenza A and B viruses, and respiratory syncytial virus, have been most strongly implicated (9, 16). The mechanism underlying this phenomenon has been studied most thoroughly with influenza A virus and *Streptococcus pneumoniae* and is believed to primarily involve viral compromise of eustachian tube mucosal integrity and function (8, 11, 12, 14, 15). The method(s) by which adenovirus predisposes a subject to bacterial OM has been less well studied because of the lack of an appropriate animal model. Recently, our laboratory reported the development of a chinchilla model of adenovirus disease which has a characteristic disease course and recovery period (4) and which uses a clinical isolate of adenovirus type 1; this model demonstrated that the chinchillas could support an active infection with this human pathogen. Chinchillas frequently presented clinical signs of disease (conjunctivitis, labored breathing, thickened secretions, and wheezing), demonstrated a 40-fold increase in complement-fixing antibody directed against the adenovirus hexon antigen, yielded a log-fold increase in viral titer in nasopharyngeal (NP) lavage fluids, had depressed ciliary activity and transport function of the tubotympanal mucosal epithelium, and showed intranuclear inclusions typical of adenovirus in mucosal epithelial cells; in addition, histopathological findings were found to correlate well with all other indicators of disease progression and recovery. Maximal compromise of the eustachian tube and middle ear epithelia, both histopathologically and functionally, occurred approximately 7 to 10 days postinoculation and required a minimum of 35 days for resolution.

To determine whether adenovirus infection could predispose subjects to nontypeable *Haemophilus influenzae* (NTHi)-induced OM, in the present study, animals were inoculated intranasally with adenovirus and NTHi either sequentially or concurrently or with the bacterial pathogen alone, and the data were compared with those obtained in the initial study, in which animals received only the viral agent. The data collectively indicated that, overall, the cohort inoculated with adenovirus and then, 7 days later, with NTHi demonstrated significantly greater signs of disease and worse damage to and a greater alteration of function of the tubotympanum than the cohort receiving the reverse order of pathogens, both pathogens simultaneously, or either agent individually. Adenovirus-induced thickened NP secretions, negative middle ear pressure, and compromised eustachian tube mucosal function are all likely to have contributed to the noted synergistic effect on NTHi-induced OM. While mucus secretions, as grossly assessed, were notably different in adenovirus-inoculated ani-

* Corresponding author. Mailing address: Otological Research Laboratories, 456 W. 10th Ave., Room 4331, Columbus, OH 43210. Phone: (614) 293-8103. Fax: (614) 293-5506. Electronic mail address: bakaletz.1@osu.edu

† Present address: Department of Otolaryngology, Nagoya City University Medical School, 1-Kawasumi, Mizuho-ku, Nagoya, 467, Japan.

mals, the data obtained in this study via lectin histochemistry did not indicate that the carbohydrate character of either the cell surface or goblet cells was markedly altered.

(This research was presented in part at the Sixteenth Midwinter Meeting of the Association for Research in Otolaryngology, 7 to 11 February 1993, St. Petersburg Beach, Fla., and the Second Extraordinary International Symposium on Recent Advances in Otitis Media, 31 March to 4 April 1993, Oita, Japan.)

MATERIALS AND METHODS

Animals. A total of 75 chinchillas (*Chinchilla laniger*; 350 to 650 g; free of middle ear disease, as determined by otoscopy) were used for these studies. Sixty-four were distributed equally among cohorts A to D, 5 were used for histopathological evaluation, and 6 were used for lectin staining. Chinchillas were chosen because of their widely accepted use as an experimental model of human OM.

Inoculations. All intranasal inoculations were performed via passive inhalation of 0.5 ml of either adenovirus type 1 (clinical isolate; a gift from David Pacini, The Children's Hospital, Columbus, Ohio) or NTHi (strain 86-028NP) suspended in sterile Eagle's minimal essential medium (MEM) (Whittaker M. A. Bioproducts, Walkersville, Md.) or saline, respectively, or with both. The dosages of viral and bacterial isolates were 6×10^6 50% tissue culture infective doses and 1×10^8 CFU per animal, respectively, with the inocula being divided equally between the nares. Two animals were inoculated per time interval per inoculation cohort. All virus-inoculated animals were housed separately from those receiving only the bacterial pathogen or until virus inoculation in those dually inoculated.

Cohort grouping. Four cohorts of 16 chinchillas each were established. Group A received adenovirus 7 days (day −7) prior to the inoculation of NTHi (day 0), group B received NTHi 7 days prior to (day −7) the inoculation of adenovirus (day 0), group C received both pathogens simultaneously (day 0), and group D received NTHi only (day 0). Animals receiving adenovirus only or sterile MEM (on day 0) (groups E and F, respectively) were part of a separate study (4) in which they were inoculated and assessed in an identical manner, unless noted otherwise.

Two animals per cohort were preassigned for sacrifice on each of days 1, 4, 7, 10, 14, 21, 28, and 35 postinoculation by lethal doses of ketamine (40 mg/kg of body weight) and xylazine (8 mg/kg) given intramuscularly. The number of days postinoculation indicated throughout this study refers to that time interval which occurred after day 0. Since our main objective was to be able to determine the effect of preexisting, and perhaps silent, NTHi colonization or adenovirus infection on the development of bacterial OM once the second agent was acquired, signs of disease present on day 0 were ignored for groups A and B, and conclusions drawn as to the severity of induced disease were based on observations made on days 1 to 35 for all cohorts.

Disease course assessment. All assessments were made blindly and by the same observer throughout this study. Animals were assessed by otoscopy daily for signs of tympanic membrane inflammation, which was rated on a scale of 0 to 4+ (0 = normal; 1+ = minimal inflammation; 2+ = moderate inflammation with a minimal effusion volume; 3+ = significant inflammation with a moderate effusion volume; and 4+ = severe inflammation, with perforation and discharge). Body temperature was determined by use of a rectal probe with a digital thermometer (Becton-Dickinson, Franklin Lakes, N.J.) on a daily basis at the same time each day with animals lightly anesthetized (2 mg of xylazine per kg intramuscularly).

On days 1, 4, 7, 10, 14, 21, 28, and 35, tympanocentesis was performed (after tympanometry [determination of middle ear pressure]) through the left tympanic membrane of one animal in each of groups A to D, and gentle negative pressure was applied to aspirate middle ear fluids (MEF), if present. The volume, color, turbidity, and viscosity of any recovered fluids were recorded. All tapped bullae were then subjected to lavage with 0.5 ml of prewarmed sterile normal saline. Lavage fluids were added to any recovered MEF, and aliquots were used for differential counting and bacterial and viral culturing. Tympanocentesis and bulla lavage were always performed before NP lavage to prevent the introduction of intranasally inoculated NTHi into bulla lavage fluids via the semipatulous eustachian tube of the chinchilla (10). Both procedures were performed on animals just prior to sacrifice; chinchillas were not multiply tapped or subjected to lavage in this study.

NP lavage was performed on two animals in each of groups A to D on days 1, 4, 7, 10, 14, 21, 28, and 35. NP lavage was additionally performed on days −5 and −3 for all animals in groups A and B (inoculated with adenovirus alone and NTHi alone on day −7, respectively) as described previously (4, 27). An aliquot of 0.4 ml of the fluids was used for the assessment of the presence of adenovirus as described below, and the remainder was used for bacterial culturing. Combined MEF and bulla lavage fluids or NP lavage fluids were cultured on chocolate agar (Choc II; BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for up to 72 h to detect and semiquantitate CFU of NTHi per milliliter (on the basis of colony morphology) or to rule out a bacterial component of any noted pathology in animals receiving the virus only. The chinchilla middle ear is sterile in healthy animals; however, the nasopharynx is typically colonized with predominantly gram-positive cocci and occasionally gram-negative bacilli (3), which are culturable on chocolate agar.

For evaluation of the numbers and types of inflammatory cells, a 50-μl aliquot of the recovered combined MEF and bulla lavage fluids was treated as previously described (21, 27). When available, 100 cells were counted at random; when less than 100 cells were present, all cells within the aliquot were counted. Counts were reported as total cells per milliliter of lavage fluid in dry ears or adjusted for the dilution effect of introduced bulla lavage fluids when MEF were present. Cytological evaluation of recovered leukocytes was limited to bulla lavage fluids because of the scarcity of inflammatory cells retrievable via NP lavage (8).

Virus identification was performed at the Clinical Virology Laboratory of The Ohio State University Hospitals, and specimens were processed within 2 to 3 h of collection. Adenovirus was preliminarily identified on the basis of characteristic cytopathic effects (cell rounding and degeneration) on A549 and NHDF cells for up to 21 days. Confirmatory identification was done with a fluorescein-tagged monoclonal antibody (Adenoclone; Cambridge Biosciences Corp., Worcester, Mass.) (4).

Light microscopic evaluation of histopathology. A separate cohort of five chinchillas (one each for groups A to D and F) was used for the evaluation of induced histopathology on day 10 postinoculation, at which time all cohorts had demonstrated maximal or near-maximal signs of induced damage. Histopathological evaluation of tympanic and pharyngeal portions of the eustachian tube and middle ear mucosae for group E was done as previously reported (4).

Dye transport. The ability of the middle ear and eustachian tube to actively transport a small volume of dye from the

middle ear to the pharyngeal orifice of the eustachian tube was assessed on both ears of one chinchilla per cohort per time point by the method of Bakaletz et al. (5). Dye transport was determined after tympanometry and NP lavage, which were predetermined to have no effect on normal transport ability in this system (3). These animals were not used for the study of ciliary activity and were not subjected to tympanocentesis. Normal transport time for chinchilla postintranasal inoculation of sterile MEM is 144 ± 8 s (4). A maximal value of 900 s was reported for all animals in which there was either no appearance of dye within an arbitrary time limit of 15 min of inoculation or an immediate appearance of dye at the pharyngeal orifice of the eustachian tube, as both situations would indicate a dysfunctional tube and/or an inability to measure active transport.

Ciliary activity. Eustachian tube mucosa obtained from the right side of one animal in each cohort at each time point was analyzed for ciliary motility by our established technique (4, 27). Five readings of ciliary beat frequency (CBF; in hertz) were averaged from three sites selected from within each of two tissue pieces procured from both the tympanic and the pharyngeal portions of the eustachian tube. Readings were taken from sites which were visually determined to have maximal ciliary activity for any given tissue piece.

Middle ear pressure. Changes in middle ear pressure were evaluated at regular intervals for each animal in cohorts A to D via tympanometry (Ear Scan; Micro Audiometric, South Daytona, Fla.). Animals in cohorts E and F were part of a previous study and were assessed prior to our use of tympanometry. Normal chinchilla middle ear pressure was considered to be between −60 daPa and +40 daPa (1, 13, 28).

Lectin histochemical examination. A separate cohort of six chinchillas was used for the investigation of potential changes in both the cell surface and the goblet cell carbohydrate characteristics of the chinchilla tubotympanum following intranasal inoculation with adenovirus type 1. We used a screening battery of 15 lectins and a lectin histochemical technique that was previously described (23). The lectins selected were *Limax flavus* agglutinin (specific for terminal NeuAc independent of the linkage pattern), *Sambuccus nigra* lectin (specific for Neu5Ac attached to terminal Gal in an α-2,6 linkage), wheat germ agglutinin (WGA) (because of both the structural similarities of *N*-acetyl neuraminic and *N*-acetylglucosamine and the high isoelectric point of the lectin, WGA binds both NeuAc and GlcNAc via their β1,4 oligomers), succinylated WGA (because of a net negative charge at a physiological pH, succinylated WGA does not bind NeuAc but retains its specificity for GlcNAc), *Bandeiraea* (*Griffonia*) *simplicifolia* II lectin (specific for α- or β-linked GlcNAc residues on the nonreducing termini of oligosaccharides, preferentially in a 1,4 linkage pattern), peanut agglutinin (specific for β-Gal, Galβ1-3Gal NAc and, to a lesser degree, Galβ1-4GlcNAc; labeling only occurs in the absence of terminal NeuAc), *Erythrina cristagalli* lectin (the highest specificity is for terminal Galβ1-4GlcNAc), *Ricinus communis* I agglutinin (specific for β-Gal, with a preference for a β-1,4 linkage, *Canavalia ensiformis* lectin (specific for α-mannose > α-Glc > α-GlcNAc), *Lens culinaris* lectin (specific for D-mannose, D-Glc, and fucose-linked α-1,6-GlcNAc), *Dolichos biflorus* lectin (specific for terminal α-D-GalNAc), *Sophora japonica* lectin (specific for GalNAc > galactose), *Phaseolus vulgaris* E lectin (specific for Galβ-1,4-GlcNAcβ-1,2-Manα-1,6), *Ulex europaeus* I agglutinin (specific for α-L-fucose and Fucα-1,2-Galβ-1,4-GlcNAc), and *Glycine max* agglutinin (specific for GalNAcα-1,3-Gal). Lectin staining was examined at tympanic, midportion, and pharyngeal sites within the eustachian tube mucosa on days 4, 10, 14, 21, 24, and 35 postinoculation of adenovirus. Controls were inoculated with sterile MEM.

Statistical analysis. An analysis of tympanic membrane inflammation scores among cohorts was done with the nonparametric method described by Koziol et al. (19), as well as with a repeated-measures analysis of variance (ANOVA), with the joint effect of cohort grouping, the block effect of time, the effect of subjects nested within cohort grouping, and the group-time interaction effect being considered. The nonparametric procedure was used to ensure that the inferences made would not be different because of the lack of normality of the data. Since the design of the experiment necessitated the sacrificing of animals at regular intervals within the 35-day observation period, the number of observations diminished over time for otoscopic scores, so the methods proposed by Milliken and Johnson (24) were used to calculate the F value and degrees of freedom, respectively, for these data only. An analysis of dye transport rates and CBF over time was done with a one-way ANOVA. Individual daily comparisons among cohorts were made with Student's t test. A P value of ≤ 0.05 was selected as the minimal level of acceptance for all analyses.

RESULTS

Clinical observations. As was observed previously (4), clinical signs of adenoviral infection were frequently noted in all cohorts receiving this agent. Incidences of approximately 30% for conjunctivitis, tympanic membrane retraction, labored breathing, thickened secretions, and lethargic and cornering behavior (animals exhibited at least one sign each) were noted. No deaths occurred in any cohort prior to sacrifice. Basal body temperature measurements were taken as an indicator of general disease state (not determined in groups E and F). There was an increase in mean temperature (1.0°F; 0.6°C) within 24 h of inoculation of either NTHi alone or both bacterial and viral pathogens delivered concurrently. Conversely, animals inoculated with adenovirus type 1 alone experienced an average decrease in body temperature of greater than 0.75°F (0.4°C) within 24 h of receiving the virus. Animals in group B (which were colonized with NTHi prior to virus inoculation) demonstrated a lesser decrease in body temperature (0.6°F; 0.3°C) within 24 h of receiving the virus.

Otoscopy. When all groups were compared over time for the severity of noted tympanic membrane inflammation (Fig. 1), cohorts which received both pathogens (groups A to C) demonstrated more severe inflammation than those which received either pathogen as a single agent. The two cohorts which received the bacterial and viral pathogens sequentially (groups A and B) were found to have significantly greater tympanic membrane changes and evidence of MEF than any of the other cohorts ($P \leq 0.005$) when compared with the MEM-inoculated control cohort (group F) in the first 10 days after inoculation of the second agent. While the plot for the group receiving adenovirus 7 days before NTHI (group A) indicated a higher average value for tympanic membrane inflammation in this group than in all the other groups for the remainder of the period of observation as well (days 10 to 35), this difference was not statistically significant. Peak tympanic membrane inflammatory changes occurred on day 10 postinoculation for groups receiving adenovirus before NTHI, both agents concurrently, or NTHi alone (groups A, C, and D, respectively), whereas this peak occurred on day 4 for groups receiving NTHi before adenovirus or adenovirus alone (groups B and E, respectively) and on day 14 for the group inoculated with sterile MEM (group F). No animals in the MEM group demonstrated ≥2+ inflammation throughout the study. The

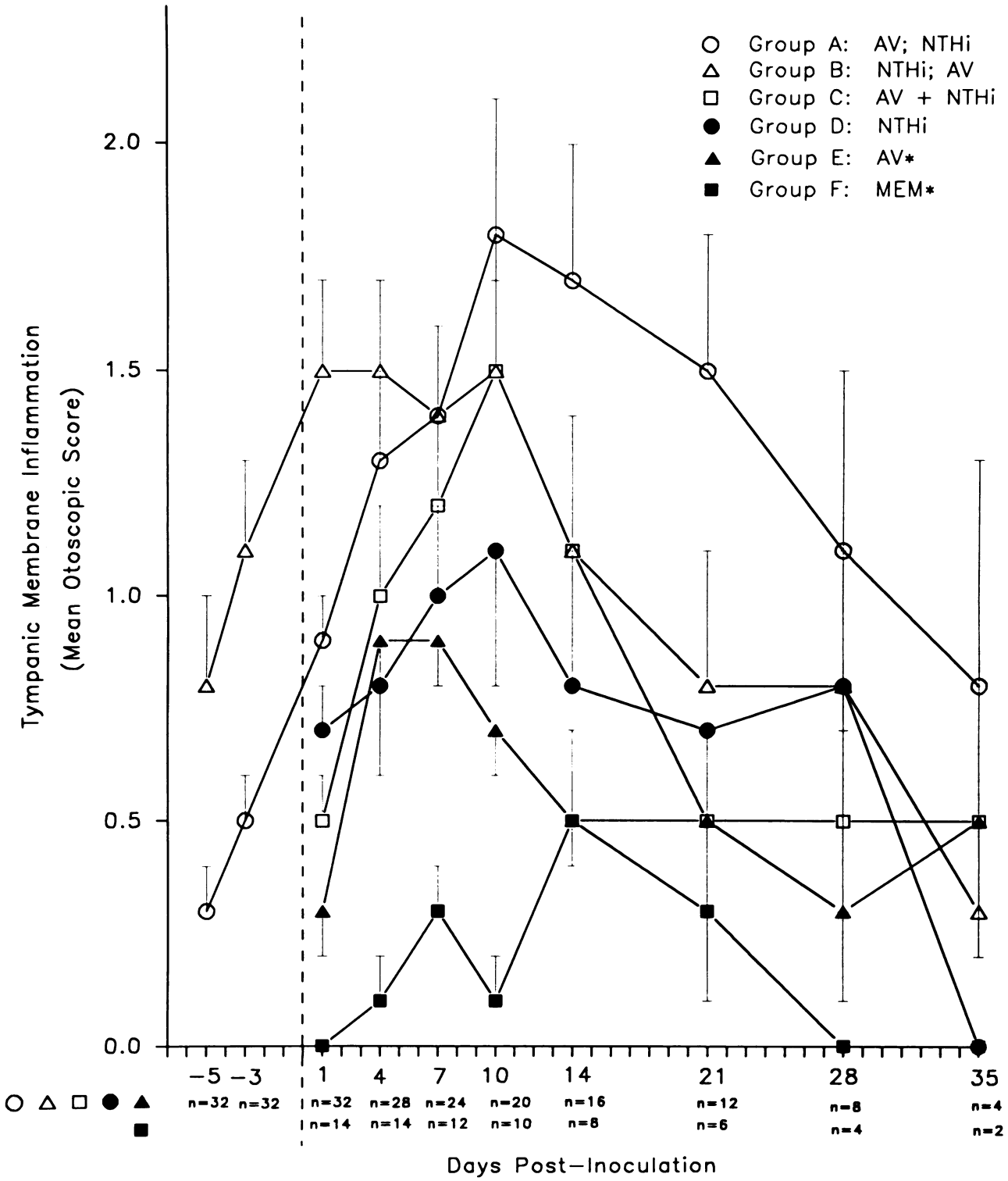

FIG. 1. Comparison among inoculation groups of mean tympanic membrane inflammation scores (± standard deviation), rated on a scale of 0 to 4+ (blindly) as determined by otoscopy over the 35-day observation period. The vertical broken line indicates day 0, when the second pathogen was inoculated for groups (A and B) which received the pathogens sequentially. *, data are from a previous study. AV, adenovirus.

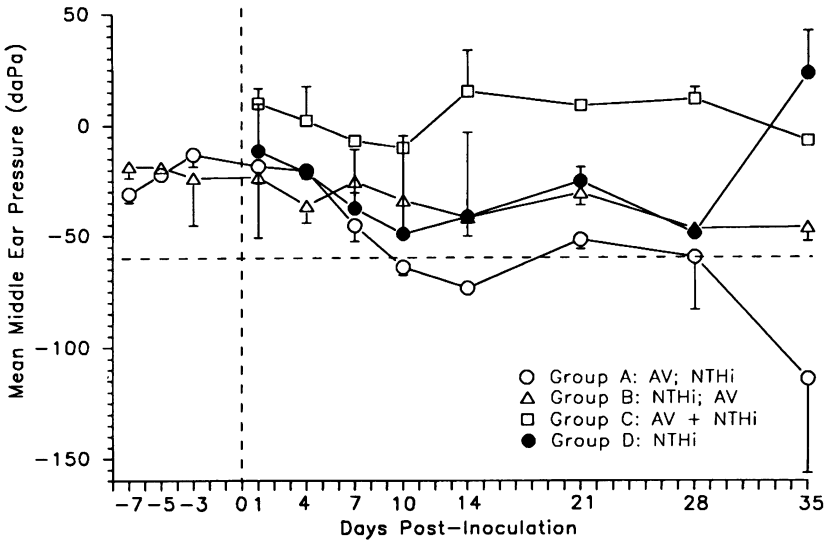

FIG. 2. Comparison among inoculation groups of mean middle ear pressure (± standard deviation), as determined by tympanometry over the 35-day observation period. The horizontal broken line indicates −60 daPa; values obtained below this line were considered abnormal for the chinchilla host (28). The vertical broken line indicates day 0, when the second pathogen was inoculated for groups (A and B) which received the pathogens sequentially. AV, adenovirus.

mild tympanic membrane inflammation noted in this cohort was likely due to a combined effect of any refluxed MEM on tympanic cavity mucosa and handling of chinchillas, as excitation will induce mild changes in the tympanic membrane.

Middle ear pressure. Tympanometry determinations were made bilaterally prior to tympanocentesis (if performed) on all chinchillas in each cohort at regular intervals for 35 days. The data indicated that significant negative middle ear pressure was a consistent finding only in animals receiving adenovirus 7 days before NTHi (group A) and occurred on days 10 to 14 and 28 to 35 (Fig. 2). No group demonstrated an abnormal positive middle ear pressure (>40 daPa) over the 35-day period. Middle ear pressure was not determined in cohorts receiving adenovirus alone or sterile MEM (groups E and F, respectively); however, retraction was noted in 89% of animals 5 days after adenovirus inoculation, whereas this sign was never noted in MEM-inoculated controls, as was previously reported (4).

Bacterial and viral cultures of lavage fluids. NTHi was not cultured from NP lavage fluids of any of 10 virus-inoculated chinchillas assayed in group A on either of the 2 days on which lavage was performed (days −5 and −3) in the week preceding the inoculation of NTHi (day 0). After the inoculation of NTHi, this organism was culturable from NP lavage fluids for up to 21 days, whereas it was culturable from bulla lavage fluids on days 14 and 21. Group B, which received an intranasal inoculation of NTHi 7 days before adenovirus, was colonized with NTHi on both days 3 and 5 prior to receiving the viral inoculum, as indicated by the fact that 10 of 10 NP lavage fluids were positive on these days. Bulla lavage fluids were culture positive only on day 1 in this cohort; however, colonization of NP lavage fluids was demonstrable to day 14. Group C, which received both pathogens concurrently, had culturable NTHi in NP lavage fluids until day 14 postinoculation and positive bulla lavage fluid cultures on days 10 and 14. Group D, which received NTHi only, had culturable NTHi in NP lavage fluids until day 14 postinoculation; however, there were no culture-positive bulla lavage fluids. Bulla lavage fluids obtained from animals receiving either adenovirus or sterile MEM (groups E and F) were predominantly bacteriologically sterile (4).

Attempts to culture adenovirus from NP lavage fluids of group A (adenovirus given 7 days prior to NTHi) in the week preceding NTHi inoculation yielded 10 of 10 positive NP lavage fluids 48 h after virus inoculation, and we previously demonstrated that adenovirus is culturable from NP lavage fluids for up to 14 days after intranasal inoculation as a single agent (4). Conversely, no virus was cultivated from any of the selected bulla lavage fluids assessed from any cohort throughout 14 days of the 35-day study period, despite the clear demonstration of intranuclear inclusions in middle ear mucosal epithelial cells after intranasal inoculation, a phenomenon which we previously reported (4). Viral culturing was not performed for cohorts which received either NTHi alone or sterile MEM (groups D and F, respectively).

Cytological evaluation of bulla lavage fluids. Lavage fluids obtained from animals inoculated with adenovirus and then NTHi (group A) demonstrated very few cells until day 14 after receiving the NTHi strain, at which time NTHi was culturable from the MEF and polymorphonuclear lymphocytes (PMNL) were the predominant cell type recovered (52%). This infiltration of PMNL was not resolved until day 28 in this cohort. Group B animals, which were colonized with NTHi prior to receiving adenovirus, had lavage fluids predominated by PMNL on day 1 (at which time the MEF were NTHi culture positive) and then lavage fluids which were predominantly acellular (≤25 countable cells) until day 14, when tympanic membrane inflammation was near maximal in this cohort. PMNL (58%) and macrophages (37%) were noted in the recovered fluids at this time; however, bulla lavage fluids obtained for the remainder of the study were acellular. For chinchillas receiving both pathogens concurrently (group C), lavage fluids contained very few cells until days 10 and 14 postinoculation, at which time NTHi was cultured from MEF and peak tympanic membrane inflammation was noted. On day 10 there was a predominantly PMNL-containing infiltrate (53%), which was gradually replaced by mononuclear cells (52%) on day 14. Bulla lavage fluids were acellular in group C for the remainder of the study. All lavage fluids obtained from animals inoculated with NTHI alone (group D) were acellular,

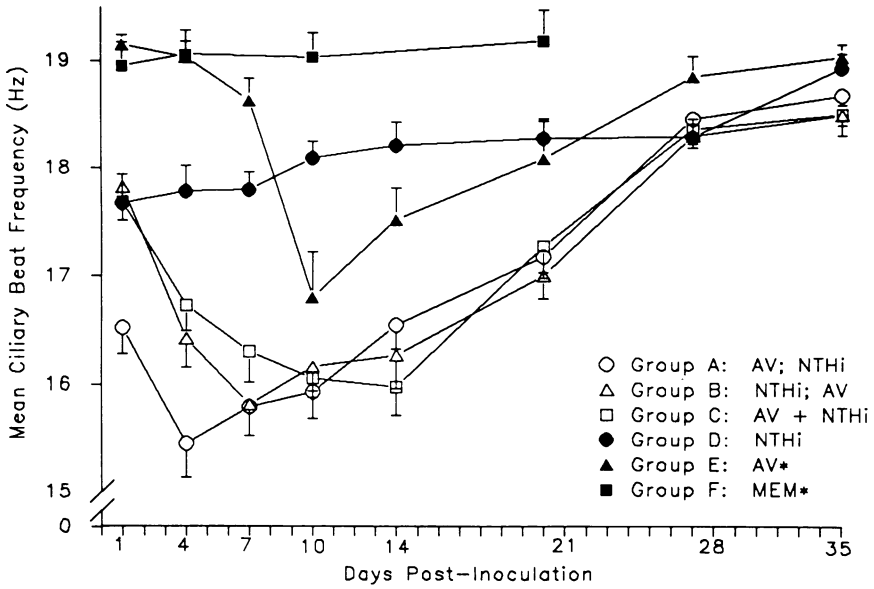

FIG. 3. Comparison among inoculation groups of mean combined CBF values (± standard deviation) for tissues from both tympanic and pharyngeal sites of the eustachian tube epithelial mucosa, as evaluated in vitro. *, data are from a previous study. AV, adenovirus.

a result which correlated well with other assessments indicating a lack of overt bacterial OM induction following intranasal inoculation of NTHi strain 86-028NP as a single pathogen. Cytological evaluation of bulla lavage fluids from adenovirus-inoculated animals (group E) has been reported (4); these fluids are typically acellular for the first 4 days postinoculation. From days 4 to 28, 67% of lavage fluids showed a primarily mononuclear infiltrate. Bulla lavage fluids recovered from group F (sterile MEM) were acellular throughout the 35-day observation period.

Ciliary activity. An in vitro determination of CBF of eustachian tube mucosa at both pharyngeal and tympanic sites was conducted at various times. Animals inoculated with sterile MEM (group F) maintained a mean CBF value of 19.2 (±0.3) Hz throughout a 21-day postinoculation period (Fig. 3). The group A (adenovirus given prior to NTHi) mean CBF value was significantly lower than that in controls throughout the observation period ($P \leq 0.001$), and the adenovirus-induced diminished CBF value was further decreased on days 1 to 4 after inoculation of the bacterial pathogen, at which time the mean CBF value was lower than that in all other cohorts ($P \leq 0.001$). From days 7 to 35 postinoculation, the plot for group A was similar to those depicted for the other two groups which received both adenovirus and NTHi (groups B and C). All groups receiving both pathogens showed a greater decrease in the mean CBF value on days 4 to 21 postinoculation than those receiving a single pathogen, although on day 10, animals receiving adenovirus alone (group E) showed a marked decrease in the mean CBF value. Group D received NTHi alone and demonstrated a lesser but significant ($P \leq 0.001$) reduction in the mean CBF value than MEM-inoculated controls throughout the study. Recovery to the baseline CBF value was not achieved on day 35 postinoculation for any group except that inoculated with sterile MEM.

Dye transport. Animals receiving sterile MEM (group F) demonstrated normal transport values over time, whereas those receiving NTHi only (group D) showed delayed transport in the first 4 days postinoculation only ($P \leq 0.001$) which rapidly resolved (Fig. 4). Animals inoculated with adenovirus prior to inoculation with NTHi (group A) demonstrated a maximal decrease in dye transport function on day 10. Group B, which was colonized with NTHi prior to inoculation with adenovirus, showed a peak suppression of dye transport function on day 10. Group C received both pathogens concurrently and also demonstrated a maximal delay of transport on day 10 which was attributable to the presence of a large volume of culture-positive MEF in this group, which prevented the determination of active transport time. Adenovirus inoculated alone (group E) was the most effective single agent with regard to the ability to result in a prolonged delay of transport; a maximal delay of transport was noted on day 10 for this group. Mean dye transport values returned to the baseline (144 ± 8 s) for all cohorts by day 35.

Histopathological findings. A histopathological assessment of the eustachian tube mucosa indicated that animals receiving sterile MEM (group F) demonstrated focal areas of subepithelial edema but overall maintained a normal appearance. Animals receiving adenovirus alone (group E) demonstrated marked goblet cell hyperplasia and focal denudement of the epithelium to the basal cell layer, with characteristic intranuclear inclusions, as we previously reported (4). Interestingly, animals receiving NTHi alone (group D) showed histopathological findings typical of experimental NTHi-induced OM (20, 26) in the eustachian tube but not the middle ear mucosa, as demonstrated by marked subepithelial edema, infiltration of inflammatory cells, disruption of ciliated cells, and osteoneogenesis. A histopathological evaluation of the eustachian tube mucosa obtained from animals in groups A, B, and C, which received both viral and bacterial pathogens either sequentially or concurrently, demonstrated effects characteristic of both NTHi and adenovirus infections: subepithelial edema, infiltration of inflammatory cells and their presence in the eustachian tube lumen, desquamation of epithelial cells, loss of cilia, focal necrosis, goblet cell hyperplasia, focal hemorrhage, submucosal thickening, new bone formation, and viral intranuclear inclusions. Similar findings were noted for the middle ear

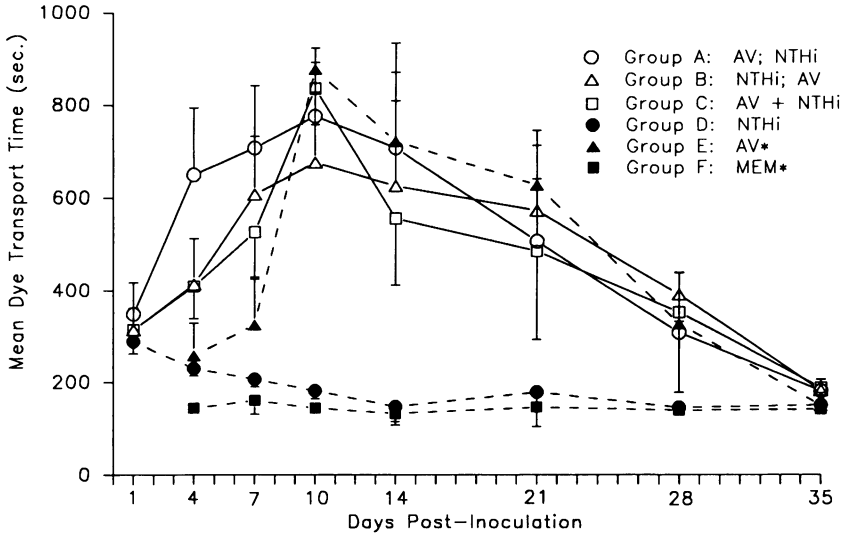

FIG. 4. Comparison among inoculation groups of the time required for middle ear and eustachian tube mucosal epithelia to transport dye, as assessed over a 35-day period. Values are expressed as mean transport time (± standard deviation). *, data are from a previous study. AV, adenovirus.

mucosa in all groups receiving both pathogens, sterile MEM, or adenovirus alone, whereas no histopathological changes were noted for the middle ear mucosa in animals receiving an intranasal inoculation of NTHi alone.

Lectin labeling. While the mucus layer itself is not maintained throughout the fixation and embedding protocol, it is produced primarily by goblet cells and glandular mucus cells and is intimately associated with the epithelial cell surface. Despite notably thickened, copious mucus and marked goblet cell hyperplasia, there were no marked differences in the lectin histochemical evaluation (as would be indicated by either a change in the intensity of lectin labeling on a scale of 0 to 3+ or a change in the distribution of the label [23]) of either the cell surface or the epithelial goblet cells within any of the three selected anatomical areas of the chinchilla eustachian tube mucosa in adenovirus-inoculated animals when compared with sterile MEM-inoculated controls for up to 35 days postinoculation with the listed battery of 15 lectins.

DISCUSSION

The association of adenovirus with bacterial OM has been recognized for a long time, yet the lack of a suitable animal model of human disease has hampered the determination of the mechanism(s) by which this predisposition may occur. The chinchilla was recently shown to support an active infection with a wild-type human adenovirus type 1 isolate obtained from a child (4). While extrapolation of the data obtained in the chinchilla to humans must be done conservatively, the chinchilla disease was found to share many characteristics with the human disease. Animals appeared to be clinically ill, often exhibiting signs of upper respiratory distress and lethargy, and demonstrated mucosal lesions typical of adenovirus as well as compromised eustachian tube mucosal integrity and function.

Because we had access to a model of adenovirus-induced upper respiratory tract disease, it was of interest to determine whether chinchillas with an active adenovirus infection were more susceptible to NTHi-induced OM than those which were not so compromised and alternatively whether animals subjected to the reverse order of pathogen acquisition (animals colonized with NTHi prior to inoculation with adenovirus) or the concurrent administration of both pathogens developed OM more readily or of increased severity. Cohorts were compared over a time course which was known to span the entire course of disease manifested by the chinchilla when adenovirus is inoculated alone (4).

The data indicated that NTHi strain 86-028NP induced inconsistent tympanic membrane inflammation in chinchillas, as determined by otoscopy. While this strain is able to readily colonize the nasopharynx after intranasal inoculation (and can indeed be cultured from the tympanum within 6 h of inoculation, likely because of passive reflux), it is cleared from the middle ear space of the majority of animals by 24 h postinoculation [6]). Intranasal inoculation of this strain did not result in culture-positive OM. There was, additionally, neither an accumulation of MEF in these animals nor a migration of inflammatory cells into the middle ear cleft, despite evidence of histopathology in the eustachian tube mucosa. Adenovirus inoculated intranasally alone also induces tympanic membrane inflammation, and the specifics of this disease course have been reported (4); however, in our experience, adenovirus-induced disease (when the virus is inoculated as a sole pathogen) has never resulted in culture-positive bacterial OM in an experimental setting. This result is likely due to the facts that chinchillas have a very low incidence of endemic OM and that, while the chinchilla nasopharynx is colonized with its own normal flora, the chinchilla is not a natural host of the primary human bacterial pathogens of OM (*S. pneumoniae*, NTHi, and *Moraxella catarrhalis*), those for which culturing is done. Thereby, the ability to culture NTHi from the bullae of dually inoculated chinchillas is indicative of the synergistic effect of these two agents.

This synergistic effect and whether it was affected by the order of pathogen acquisition were the focus of this study. Animals which were inoculated with adenovirus 7 days prior to receiving NTHi were the most affected cohort overall. At the time of NTHi inoculation, these animals were demonstrating middle ear pressure values within normal limits, with minimal mean tympanic membrane inflammation; however, the mean

CBF was markedly reduced ($P \leq 0.001$) compared with those in all other cohorts. Within 4 days of inoculation of NTHi, CBF values had further decreased to their maximally diminished levels; middle ear pressures became more negative, and both tympanic membrane inflammation scores and dye transport times increased. The pronounced and sustained negative middle ear pressures recorded in this dual-pathogen-inoculated cohort indicated that virus-induced effects were influencing this parameter, as NTHi-induced OM typically results in culture-positive MEF and an abnormally positive middle ear pressure. The net effect of these two pathogens was induced negative middle ear pressure, an effect which was also grossly observed as "retraction" upon otoscopy. Elevated mean tympanic membrane inflammation scores were the most prolonged in this cohort as well.

Our previous study (4) showed that chinchillas inoculated with adenovirus demonstrated the most severe signs of disease (by all methods of assessment) at approximately the time at which NTHi would have been introduced in this cohort. Factors contributing to the noted synergistic effect were likely the previously demonstrated adenovirus-induced negative middle ear pressure; middle ear and eustachian tube epithelial cell histopathology; and goblet cell hyperplasia, with resultant increases in both the amount and the viscosity of mucus. There are multiple possible factors contributing to the noted gross changes in the amount and viscosity of mucus overlying the tubotympanal mucosa in adenovirus-inoculated animals. There was, however, no notable change in either the epithelial cell surface or the goblet cell carbohydrate character when 15 lectins selected for this study were used, indicating that perhaps these animals were experiencing hypersecretion of "normal" mucus and an inability to adequately hydrate the mucus gel. These lectin data are similar to those obtained after transbullar inoculation of NTHi but are in contrast to those obtained after either transbullar or intranasal inoculation of *S. pneumoniae*; in the latter case, the resultant OM induces a distinct and marked change in the lectin labeling pattern, presumably because of the effect of pneumococcal enzymes which reveal moieties of their own receptors (22, 23). Regardless of the mechanism(s) which resulted in the thickened and copious mucus, altered mucus rheological properties affected mucociliary function, and this factor did appear to contribute to the predisposition to bacterial OM in this model.

Animals which were demonstrated to have NTHi-colonized nasopharynges prior to receiving the viral pathogen (group B), while maintaining colonization throughout the majority of the study period and clearly demonstrating altered eustachian tube mucosal function, were not the most adversely affected group. At the time of receipt of the viral pathogen, the nasopharynx was colonized with NTHi, and both eustachian tube transport function and middle ear pressure were within normal limits; however, there was inflammation of the tympanic membrane. This inflammation may explain, in part, the milder disease seen in these animals, as viral clearance may have been augmented by the existing inflammatory process. While these data do not provide support for the generally held belief that children are first colonized with NTHi, contract a viral "cold," and then develop bacterial OM, as in middle and lower respiratory tract infections caused by *H. influenzae* (18), as a result of viral compromise of host defense mechanisms (2, 7, 16, 17), the results obtained here were probably due to the model system used. In our model, inoculation of adenovirus 7 days after intranasal inoculation of NTHi was chosen to best capitalize on the adenovirus-induced compromise of the chinchilla tubotympanum to induce OM by intranasal inoculation of NTHi but may not have been the optimal time interval for modeling synergy in the reverse order of acquisition. Determination of the optimal time period for demonstrating synergy between NP colonization by NTHi and then adenovirus inoculation, as has been done for pneumococci and influenza A virus by Abramson et al. (1), will require further study. It is clear, however, that NP colonization of children with NTHi is a dynamic process in which children acquire, lose, and reacquire different NTHi strains with great regularity (25). When, in this dynamic process, the viral agent which ultimately predisposes them to bacterial OM is contracted is not currently known.

Animals receiving both pathogens concurrently (group C) demonstrated the expected NP colonization by the bacterial pathogen and were also more adversely effected than those receiving either of the pathogens alone. There was, however, no evidence of a marked alteration of middle ear pressure throughout the observation period, and tympanic membrane inflammation resolved more quickly than in either of the other two cohorts which had received the two pathogens sequentially. Inhibition and subsequent recovery of both normal CBF and dye transport were, however, very similar to those noted for the other two dually inoculated groups. It is not clear from our data what accounted for the overall diminished induction of OM noted for this group; however, it is possible that by the time adenovirus-induced damage was optimal for predisposition to bacterial OM originating from the NTHi-colonized nasopharynx, combined host defense mechanisms prevented disease induction.

In conclusion, in addition to providing support for epidemiological studies which have long demonstrated the existence of adenovirus-caused predisposition to bacterial OM, this study also provides a new method of modeling OM in the chinchilla. While a traditional transbullar inoculation model has been useful for modeling various aspects of human disease, there are limitations to the interpretation of findings obtained in this manner, as the method of pathogen acquisition is not analogous to that which occurs in humans. Intranasal inoculation of NTHi, while a more natural method of pathogen introduction, is a very strain-dependent model (6) and does not result in either significant disease or consistent induction of disease in the chinchilla. This inconsistent disease induction does not readily allow for a statistical comparison of induced OM between cohorts, nor does it allow readily for a comparison of the efficacies of potential vaccine or intervention strategies among strains.

The use of a model in which adenovirus disease is induced prior to the intranasal inoculation of NTHi results in consistent and progressive development and subsequent resolution of OM in the chinchilla. Furthermore, existing epidemiological evidence supports the use of this dual inoculation model. This model, with its exclusive use of intranasal inoculation, will allow us to more effectively and accurately model human bacterial OM as the organism moves from the initial inoculation site in the nasopharynx to the middle ear via the eustachian tube in a progressive fashion that requires several days to become established. This model will also be useful in future assessments of the effects of various vaccine regimens or intervention strategies (e.g., the use of antiadhesive agents) as well as in defining the optimal parameters for their use during disease progression and resolution. It provides us with the ability to monitor not only the status of NP and middle ear colonization but also traditional clinical indicators of OM, histopathological changes, and functional correlates of noted histopathological changes.

While only the effect of adenovirus type 1 and NTHi on tubotympanal mucosal integrity and function was assessed in this study, there are surely additional systemic effects which

contribute to the overall disease course noted in the chinchilla. Viral effects on phagocyte migration and function, cytokine production, lymphocyte recruitment, and various components of complement, among others, will need to be determined to fully understand the pathogenesis of adenovirus-augmented NTHi disease in this host.

ACKNOWLEDGMENTS

This study was supported by a grant from NIDCD/NIH (DC 00090) and by an O.S.U. College of Medicine development fund (536326).

We gratefully acknowledge the contributions of Grace Rees for manuscript preparation, Valerie Jones for creation of photographs and figures, and Beth McQuire and Brian Hiestand for technical assistance. We thank G. Scott Giebink and Pearay Ogra for critical review of the manuscript.

REFERENCES

1. **Abramson, J. S., G. S. Giebink, and P. G. Quie.** 1982. Influenza A virus-induced polymorphonuclear leukocyte dysfunction in the pathogenesis of experimental pneumococcal otitis media. Infect. Immun. **36:**289–296.
2. **Arola, M., O. Ruuskanen, T. Ziegler, J. Mertsola, K. Näntö-Salonen, A. Putto-Laurila, M. Viljanen, and P. Halonen.** 1990. Clinical role of respiratory virus infection in acute otitis media. Pediatrics **86:**848–855.
3. **Bakaletz, L. O.** Unpublished data.
4. **Bakaletz, L. O., R. L. Daniels, and D. J. Lim.** 1993. Modeling adenovirus type 1-induced otitis media in the chinchilla: effect on ciliary activity and fluid transport function of eustachian tube mucosal epithelium. J. Infect. Dis. **168:**865–872.
5. **Bakaletz, L. O., S. R. Griffith, and D. J. Lim.** 1989. The effect of prostaglandin E_2 and bacterial endotoxin on the rate of dye transport in the eustachian tube of the chinchilla. Ann. Otol. Rhinol. Laryngol. **98:**278–282.
6. **Bakaletz, L. O., T. M. Hoepf, D. J. Lim, and B. Tallan.** 1990. Colonization of the chinchilla middle ear and nasopharynx by fimbriated isolates of nontypable *Haemophilus influenzae*, abstr. B-66, p. 37. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990.
7. **Chonmaitree, T., V. M. Howie, and A. L. Truant.** 1986. Presence of respiratory viruses in middle ear fluids and nasal wash specimens from children with acute otitis media. Pediatrics **77:**698–703.
8. **Chung, M. H., S. R. Griffith, K. H. Park, D. J. Lim, and T. F. DeMaria.** 1992. Cytological and histological changes in the middle ear after the inoculation of influenza A virus. Acta Otolaryngol. (Stockholm). **113:**81–87.
9. **Clements, D. A., F. W. Henderson, and E. C. Neebe.** 1993. The relationship of viral isolation to otitis media in a research day care center 1978–88, p. 27–29. *In* D. J. Lim, C. D. Bluestone, J. O. Klein, D. J. Nelson, and P. L. Ogra (ed.), Proceedings of the Fifth International Symposium on Recent Advances in Otitis Media. B. C. Decker, Inc., Hamilton, Ontario, Canada.
10. **Doyle, W. J.** 1985. Eustachian tube function in the chinchilla. Arch. Otolaryngol. **111:**305–308.
11. **Giebink, G. S., I. K. Berzins, S. C. Marker, and G. Schiffman.** 1980. Experimental otitis media after nasal inoculation of *Streptococcus pneumoniae* and influenza A virus in chinchillas. Infect. Immun. **30:**445–450.
12. **Giebink, G. S., I. K. Berzins, G. Schiffman, and P. G. Quie.** 1979. Experimental otitis media in chinchillas following nasal colonization with type 7F *Streptococcus pneumoniae*: prevention after vaccination with pneumococcal capsular polysaccharide. J. Infect. Dis. **140:**716–723.
13. **Giebink, G. S., K. A. Heller, and E. R. Harford.** 1982. Tympanometric configurations and middle ear findings in experimental otitis media. Ann. Otol. Rhinol. Laryngol. **91:**20–24.
14. **Giebink, G. S., M. L. Ripley, and P. F. Wright.** 1987. Eustachian tube histopathology during experimental influenza A virus infection in the chinchilla. Ann. Otol. Rhinol. Laryngol. **96:**199–206.
15. **Giebink, G. S., and P. F. Wright.** 1983. Different virulence of influenza A virus strains and susceptibility to pneumococcal otitis media in chinchillas. Infect. Immun. **41:**913–920.
16. **Henderson, F. W., A. M. Collier, M. A. Sanyai, et al.** 1982. A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion. N. Engl. J. Med. **306:**1377–1383.
17. **Klein, J. O., and D. W. Teele.** 1976. Isolation of viruses and mycoplasmas from middle ear effusions: a review. Ann. Otol. Rhinol. Laryngol. **85:**140–144.
18. **Korppi, M., M. L. Katila, J. Jaaskelainen, and M. Leinonen.** 1992. Role of non-capsulated *Haemophilus influenzae* as a respiratory pathogen in children. Acta Paediatr. (Stockholm) **81:**989–992.
19. **Koziol, J. A., D. A. Maxwell, M. Fukushima, M. E. M. Colmerauer, and Y. H. Pich.** 1981. A distribution-free test for tumor growth curve analyses with applications to an animal tumor immunotherapy experiment. Biometrics **37:**383–390.
20. **Lewis, D. M., J. L. Schram, S. J. Meadema, and D. J. Lim.** 1980. Experimental otitis media in chinchillas. Ann. Otol. Rhinol. Laryngol. **89**(Suppl. 68)**:**344–350.
21. **Lim, D. J., D. M. Lewis, J. L. Schram, and H. G. Birck.** 1979. Otitis media with effusion: cytological and microbiological correlates. Arch. Otolaryngol. **105:**404–412.
22. **Linder, T. E., R. L. Daniels, D. J. Lim, and T. F. DeMaria.** The effect of intranasal inoculation with *Streptococcus pneumoniae* on the carbohydrate structure of the eustachian tube and middle ear mucosa in chinchillas. Submitted for publication.
23. **Linder, T. E., D. J. Lim, and T. F. DeMaria.** 1992. Changes in the structure of the cell surface carbohydrates of the chinchilla tubotympanum following *Streptococcus pneumoniae*-induced otitis media. Microb. Pathog. **13:**293–303.
24. **Milliken, G. A., and D. E. Johnson.** 1992. Analysis of messy data, vol. 1. Designed experiments. Van Nostrand Reinhold, New York.
25. **Murphy, T. F., J. M. Bernstein, D. M. Dryja, A. A. Campagnari, and M. A. Apicella.** 1987. Outer membrane protein and lipooligosaccharide analysis of paired nasopharyngeal and middle ear isolates in otitis media due to nontypable *Haemophilus influenzae*: pathogenetic and epidemiological observations. J. Infect. Dis. **156:**723–731.
26. **Okazaki, N., T. F. DeMaria, B. R. Briggs, and D. J. Lim.** 1984. Experimental otitis media induced by nonviable *Haemophilus influenzae*: cytological and histological study. Am. J. Otolaryngol. **5:**80–92.
27. **Park, K. H., L. O. Bakaletz, J. Coticchia, and D. J. Lim.** 1993. Effect of influenza A virus infection on ciliary activity and dye transport function in the chinchilla eustachian tube. Ann. Otol. Rhinol. Laryngol. **102:**551–558.
28. **Rosenfeld, R. M., W. J. Doyle, D. Swarts, J. Seroky, and B. P. Pinero.** 1992. Third-generation cephalosporins in the treatment of acute pneumococcal otitis media. An animal study. Arch. Otolaryngol. Head Neck Surg. **118:**49–52.