FUNCTIONAL AND ULTRASTRUCTURAL EFFECTS OF NONTYPEABLE HAEMOPHILUS INFLUENZAE IN A HAMSTER TRACHEA ORGAN CULTURE SYSTEM

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SUMMARY

A hamster trachea organ culture system was utilized to evaluate quantitatively the effects of a strain of nontypeable Haemophilus influenzae (NTHI) and culture supernatants of the same strain on ciliary activity. Tracheal explants were maintained in organ culture for 96 to 144 h and ciliary activity was observed daily with an inverted microscope. Explants continuously exposed to a strain of NTHI had a progressive decline in ciliary activity which was significantly lower than uninfected controls evaluated concomitantly by 48 h of exposure and thereafter. Histologic studies revealed a progressive degeneration of mucosal cells and exfoliation of ciliated cells. Scanning electron microscopy showed little adherence of NTHI to the mucosal surface. Sterile broth cultures of NTHI and supernatants of organ cultures infected with the same NTHI strain had no adverse effect on ciliary activity. Infected tracheal explants treated with ampicillin 24, 48, or 72 h after continuous bacterial challenge had no significant decline in ciliary activity compared to controls. The lack of adherence and the histologic changes observed when hamster trachea cultures were infected with NTHI suggested a toxin might mediate the damage observed. Broth and organ culture supernatants, however, produced no damage. Therefore, further studies are needed to determine the role, if any, of a toxin in the production of damage to hamster tracheal explants by NTHI.

Key words: nontypeable H. influenzae; trachea organ culture; respiratory infection.

INTRODUCTION

Nontypeable Haemophilus influenzae (NTHI) is found frequently in sputum cultures of patients with chronic bronchitis during acute exacerbations (12,13,15). This has led to the use of antimicrobial therapy directed at the NTHI during exacerbations. The potential pathogenicity of the organism is unquestioned because NTHI has been found to produce invasive disease with associated bacteremia in adults (5,20). However, the importance of this organism and its role in causing acute exacerbations of chronic bronchitis has been questioned (18).

The controversy concerning the role of NTHI in producing exacerbations of chronic bronchitis is compounded by the minimal information available regarding the pathogenesis of respiratory infection due to this organism. Denny studied the effects of H. influenza b and NTHI on trachea organ cultures from several animal species (6). He observed that NTHI produced "ciliostasis" of the tracheal epithelium at varying rates which was species dependent. Of the species tested rat tracheal epithelium was the most sensitive tissue. Broth culture supernatants of H. influenzae had a similar ciliostatic effect on rat trachea cultures. Biochemical studies of the culture supernatant suggested the ciliostatic substance was lipopolysaccharide (LPS). No further studies to our knowledge have been published verifying the ciliostatic substance as LPS. More recently Johnson et al. (11) have found by scanning electron microscopy that NTHI adhere minimally to rat tracheal epithelium.

We have developed a hamster trachea organ culture system to study the pathogenesis of NTHI respiratory infection. This particular system was chosen because it has been used successfully to study the pathogenesis of respiratory infection due to Mycoplasma pneumoniae and other bacteria (2-4,8). These studies, which have produced considerable information concerning the mechanisms by which these organisms damage respiratory epithelium, served as a guideline for our studying NTHI.

The objectives of the present study were to utilize the trachea organ culture system to evaluate (a) the pathogenic effect of NTHI and culture supernatants of this organism on respiratory epithelium by quantitating their effect on ciliary activity and by histologic studies and (b) the impact of antibiotic treatment of infected organ cultures on the pathogenic process.
Materials and Methods

Bacteria. A sputum isolate, NTHI 7891, was obtained from a patient with chronic bronchitis and pneumonia. It was identified as *Hemophilus influenzae* by colonial morphology and requirement for X and V factors. This isolate was determined to be nontypeable by counter-immunoelectrophoresis as previously described (14). Stock cultures were maintained in defibrinated sheep blood at −70°C. To prepare inocula, cultures were incubated at 37°C in 5% CO₂ in Schaedler’s broth (Difco, Detroit, MI) supplemented with Fildes enrichment (Difco).

Media. Organ cultures were maintained in Eagle’s minimal essential medium (MEM) with Earle’s salts and L-glutamine (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO). For experiments utilizing broth or organ culture supernatants no fetal bovine serum was added to the MEM. During the initial preparation of the organ cultures and for 24 h thereafter, MEM was supplemented with potassium penicillin G (200 U/ml; E. R. Squibb and Sons, Inc., Princeton, NJ) and vancomycin hydrochloride (5 μg/ml; Eli Lilly and Co., Indianapolis, IN).

Preparation of hamster trachea organ cultures. Tracheas from adult male and female Syrian hamsters (Charles River Breeding Laboratories, Wilmington, MA) were obtained using the method described by Gabridge (9). Hamsters were anesthetized with an intraperitoneal injection of 25 mg of pentobarbital. After suitable anesthesia was achieved, the hamster was exsanguinated by cardiac puncture and the trachea was removed and sectioned into rings of tissue measuring approximately 1 mm in thickness and 2 mm in diameter. The explants were washed in MEM and placed (two per dish) in tissue culture-grade plastic petri dishes (35 × 10 mm; Falcon Plastics, Oxnard, CA), covered with 0.7 ml of MEM containing penicillin and vancomycin, and incubated at 37°C in 5% CO₂. Twenty-four hours after the organ cultures were established, explants were examined for ciliary activity (see below). Explants found to have no ciliary activity were transferred to new petri dishes (two explants per dish) and MEM without bovine serum in order to have enough volume of each supernatant to inoculate explants daily for 4 d.

Preparation of culture supernatants. NTHI from stock culture was grown overnight in flasks containing 100 ml of Schaedler’s broth supplemented with Fildes’ enrichment at 37°C in 5% CO₂. After overnight growth, broth cultures were handled in several different ways in regard to sterilization of the culture. One 100-ml aliquot of broth culture was centrifuged at 10,000 g at 4°C for 20 min, the supernatant decanted into sterile tubes, and then passed through a 0.45 μm filter (Acrodisc, Gelman). Another 100 ml aliquot was centrifuged as above and ampicillin added (final concentration, 100 μg/ml) to the decanted supernatant and this supernatant was incubated at 37°C overnight. A third 100-ml aliquot of broth culture was not centrifuged and ampicillin was added to the culture to give a final concentration of 100 μg/ml and this culture was reincubated at 37°C overnight. Two other 100-ml aliquots of broth culture were similarly handled as just described with ampicillin except chloramphenicol (final concentration, 50 μg/ml) was substituted. As a control, sterile Schaedler’s broth plus Fildes’ enrichment was incubated overnight at 37°C in 5% CO₂, centrifuged at 10,000 g at 4°C, filter sterilized, subcultured to ensure sterility, and frozen at −20°C. All the other aliquots of supernatants were checked for sterility by subculture on chocolate agar. Once sterility was verified, all supernatant preparations were frozen at −20°C until used. Before inoculation of tracheal explants, all supernatants were diluted 1:5 in MEM without fetal bovine serum.

Organ culture supernatant was prepared from tracheal explants infected with NTHI 7891 as follows. MEM from explants infected for 24, 48, 72, and 96 h was placed in individual sterile test tubes (one tube for each dish) and frozen at −20°C. After it was determined by subculture that these aliquots of MEM contained only NTHI, they were pooled into two separate aliquots. One aliquot was sterilized by passing through a 0.45 μm filter. The other aliquot was sterilized by addition of ampicillin (100 μg/ml). The pooled supernatants were subcultured on chocolate agar to verify sterility and frozen at −20°C until used. MEM was pooled from several experiments to obtain an adequate volume for testing. MEM from uninfected control explants was obtained at the same time intervals, subjected to the same manipulations as infected MEM, and frozen at −20°C until used. For testing in the hamster model all organ culture supernatants had to be diluted 1:3 with MEM without fetal bovine serum in order to have enough volume of each supernatant to inoculate explants daily for 4 d.

Assessment of functional damage. Functional damage to hamster tracheal explants was assessed by measuring ciliary vigor and determining the percentage of the circumference of the mucosa with ciliary activity (PCCA) daily during an experiment (9). Ciliary vigor and PCCA were determined by examination of explants with an inverted microscope at a magnification of ×100 to 200. Ciliary vigor was graded on a scale of 0 to 4: 4, very fast, coordinated beating; 3, fast beating; 2, slow beating; 1, feeble, disorganized beating; 0, inactive. Explants were chosen for an experiment only if the PCCA was 75% or more and the ciliary vigor was 4+. The relative activity index of an explant was calculated by multiplying together values for ciliary vigor and PCCA (9). The relative activity index of an explant at each time of observation was expressed as a percentage of the initial index using the following formula: Percent relative activity index remaining = Relative activity index at each time of observation / Relative activity index just before bacterial challenge (t = 0) × 100.

Organ culture dishes were coded and previous scores for ciliary vigor and PCCA were not known by observers at the time of each assessment. Each explant was independently assessed daily by two observers. The activity index of an explant at each time period of