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An Animal Model Demonstration of Enhanced Clearance of Nontypable *Haemophilus influenzae* from the Respiratory Tract after Antigen Stimulation of Gut-associated Lymphoid Tissue[1-3]

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SUMMARY An animal model of the clearance of nontypable *Haemophilus influenzae* has been developed to determine both optimal immunization strategies for controlling colonization of the respiratory tract in patients with damaged airways and mechanisms of action of immune clearance. It was demonstrated that stimulation of gut-associated lymphoid tissue (GALT) (either by direct injection or by ingestion of antigen) followed by local administration of antigen into the bronchus was required to enhance clearance in this model. The primary effect of GALT immunization persisted for at least 6 wk; it was specific and could not be replaced by systemic immunization. Failure to stimulate locally in the bronchus was associated with protracted clearance. No clear correlation between local or systemic antibody and bacterial clearance was demonstrated; however, immunized rats were shown to have faster recruitment of phagocytic cells to the bronchial spaces, and these phagocytes had a higher activity state than cells harvested from nonimmunized animals. It is probable that bacterial clearance is accelerated in immunized animals due in part to factors mediating a change in the behavior of luminal phagocytes. AM REV RESPIR DIS 1989; 140:311-316

Introduction

Nontypable *Haemophilus influenzae* (NTHI) has been implicated as a major etiologic agent in the pathogenesis of the acute bronchitis that complicates the course of chronic bronchitis (1). A killed preparation of NTHI, when administered orally, has been shown to reduce the frequency of these acute episodes (2). The mechanism of action of this vaccine, however, remains speculative. To study the relative efficiency of different routes of immunization and mechanisms responsible for clearance of bacteria from the respiratory tract, a rat model has been established in which killed bacteria are presented to the gut-associated lymphoid tissue (GALT) of healthy rats. Measurement of the rate of pulmonary clearance of NTHI has been used to evaluate the protective value of the vaccine. The mechanism of action has been studied by noting the rate of recruitment and activity state of luminal phagocytes. In addition, the role of antibody has been investigated by evaluation of the level of NTHI-outer membrane protein (OMP)-specific antibodies in serum, saliva, and bronchial washings of immunized rats in an ELISA assay.

Methods

Rats

Male and female DA rats were maintained in a specific pathogen-free environment throughout the experiment. Immunization protocols were commenced at 6 to 8 wk of age.

Immunization

Nontypable *H. influenzae* (biotype I) was grown overnight on chocolate agar at 37° C in a candle extinction jar. Bacteria were harvested after 16 h and suspended in sterile phosphate buffer pH 7.3 containing 0.15M NaCl (phosphate-buffered saline [PBS]). Purity was checked by replating for single colony growth on chocolate agar. The bacteria were killed by incubating overnight at 4° C in an equal volume of 2% (wt/vol) formaldehyde in PBS. The preparation was washed four times in sterile PBS and resuspended to a final concentration of 2×10^{10} colony-forming units (cfu)/ml (assessed by optical density at 405 nm on a Titertek Multiskan spectrophotometer).

Intra-Peyer's patch (IPP) inoculations were performed under Saffan anesthesia by subserosal injection through a 27 g needle as described by Husband and Dunkley (3). In some regimes, the NTHI suspension was emulsified in an equal volume of Freund's complete adjuvant (FCA) to a final concentration of 1×10^{10} cfu/ml. In other regimes, the NTHI suspension was diluted in sterile PBS to 1×10^{10} cfu/ml. The preparation was distributed in 2 to 5 µl volumes above each Peyer's patch. Oral inoculations were carried out via gastric intubation under ether anesthesia by the administration of 0.5 ml of 1×10^{9} cfu NTHI in an equal volume of saturated $NaHCO_3$. Intratracheal inoculations were carried out under ether anesthesia by instillation of 50 µl PBS containing 1×10^{10} cfu NTHI into the lungs via a 20 g cannula placed intratracheally. Subcutaneous injections were given at the back of the neck with 1×10^{9} cfu NTHI in a 0.5 ml volume.

Inoculation Regimes

The ability of the killed vaccine to give clinical protection was studied under a number of different regimes summarized in table 1. In Regimes 1 to 8, the priming dose was given on Day 0, followed by boosting on Days 14 and 17, with clearance assessment on Day 21. The chosen schedule was aimed to coincide intratracheal boosting with time of peak migration of antibody-containing cells from the GALT after GALT immunization, which was established by Pierce and Gowans in 1975 (4). In two long-term studies, boosting doses were administered after 5 (Regime 10) and 8 wk (Regime 11) respectively, with clearance assessment the following week. In Regime 9, the vaccine was administered orally for 2 wk leading up to, and in place of, the standard priming inoculation. In each regime, control rats were given PBS by the same route at each time point.

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TABLE 1

IMMUNIZATION REGIMES AND RESULTS OF CLEARANCE RATE ASSESSMENT

Regime	Priming Dose		Boost		Number	Mean Number of cfu in Lungs*	p Value
Nonimmunized	PBS		PBS		38	6.58 ± 0.16	
1	NTHI + FCA	IPP	NTHI	IT	6	4.08 ± 0.25	< 0.05
2	NTHI	IPP	NTHI	IT	6	5.11 ± 0.34	< 0.05
3	FCA	IPP	PBS	IT	5	6.14 ± 0.52	> 0.05
4	PBS	IPP	NTHI	IT	5	6.66 ± 0.34	> 0.05
5	NTHI	IT	NTHI	IT	5	6.41 ± 0.13	> 0.05
6	NTHI + FCA	IPP	PBS	IT	6	7.42 ± 0.19	< 0.05
7	NTHI	IPP	PBS	IT	6	7.87 ± 0.15	< 0.05
8	NTHI	SC	NTHI	IT	5	6.18 ± 0.55	> 0.05
9	NTHI	PO	NTHI	IT	6	4.41 ± 0.37	< 0.05
10 (a)	NTHI	IPP	NTHI	IT	5	5.27 ± 0.36	< 0.05
(b)	NTHI + FCA	IPP	NTHI	IT	5	5.34 ± 0.34	< 0.05
11 (a)	NTHI	IPP	NTHI	IT	6	6.72 ± 0.15	> 0.05
(b)	NTHI	IPP	NTHI	IT	6	6.43 ± 0.22	> 0.05

Definition of abbreviations: cfu = nontypable *Haemophilus influenzae* colony-forming units; PBS = phosphate-buffered saline; NTHI = nontypable *H. influenzae*; FCA = Freund's complete adjuvant; IPP = intra-Peyer's patch; IT = intratracheal; SC = subcutaneous; PO = per os.

* Values are mean ± SE.

Rats were immunized according to the above regimes with priming dose at Day 0, in Regimes 1-8, boosts on Days 14 and 17, and clearance rate assessment on Day 21. In Regime 9, the vaccine was administered orally for 2 wk leading up to, and in place of, the standard priming inoculation. In Regime 10, boosts were given on Days 35 and 38 with clearance rate assessment on Day 42; in Regime 11, boosts were given on Days 56 and 59 with clearance rate assessment on Day 63. In all groups, control rats were given PBS by the same route at each time point. Mean numbers of colony-forming units remaining in lungs are expressed in $\log_{10}$ values. *T* tests were performed on each immunized group with respect to the nonimmunized group, and p values of less than 0.05 were considered significant.

Clearance Assay

At the end of each inoculation regime, rats were intratracheally administered a suspension of 10^8 to 10^9 cfu live NTHI harvested from an overnight culture. Four hours later bronchial washings were collected by five sequential lavages with 100 U/ml heparin in PBS to obtain a final volume of 10 ml. The number of cfu remaining in the lungs was calculated by serial dilution and plating of a sample of the lavage on chocolate agar. The number of cfu that grew overnight was corrected for volume to provide an indication of absolute numbers of cfu remaining in the lungs. The values for nonimmunized rats remained similar throughout the experiment, and a mean of the 38 control values was calculated (6.58 ± 0.16 $\log_{10}$ cfu). Variation from this control mean was used as a basis for comparing immunization regimes.

Bronchoalveolar Lavage Cells

One hundred microliters was taken from each bronchial lavage sample from Regimes 2, 5, 7, and 8. These regimes include the systemic regime (Regime 8), two mucosal regimes, one conferring protection (Regime 2) and one not conferring protection (Regime 5), and a regime that retards clearance (Regime 7). Samples were spun at 10 × *g* for 4 min, and harvested cells were stained with the Romanowsky stain azure B/eosin Y (5) for differential cell counts. The remainder of the lavage was centrifuged at 400 × *g* for 10 min. The supernatant was retained for antibody determination. The cell pellet was washed and resuspended in Hank's balanced salt solution (HBSS) (phenol free) to a final concentration of 1.25 × 10^6 cells/ml. Viability of cells was measured by trypan blue exclusion.

Chemiluminescence

Bronchoalveolar lavage (BAL) phagocytic cell activation was gauged by measurement of luminol-induced chemiluminescence. Cells were maintained at 37° C with continuous mixing in a Wallac luminometer (Model 1251; LKB, Stockholm, Sweden). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was prepared as a stock solution in DMSO (0.002 M). This was diluted 1:250 in HBSS prewarmed to 37° C for the assay. The concentration of luminol used was based on data compiled by Allred and colleagues (6) that showed a peak in enhancement of chemiluminescence when luminol was present in solution at concentrations of 10^{-6} to 10^{-4} M. Zymozan was prepared as a stock solution by boiling in saline at 10 mg/ml for 10 min, washing twice in PBS, and resuspending to 80 mg/ml in PBS. Stock zymosan was diluted 1:4 in PBS and dispersed by repeated passage through a 26 g needle. An equal volume of rat serum diluted 1:10 in PBS was added, and the mixture was incubated at 37° C for 30 min in a shaking waterbath. After centrifugation for 10 min at 1600 × *g*, the pellet was collected and washed in PBS before dilution in HBSS to a concentration of 10 mg/ml.

Regimes tested for enhanced phagocyte activity were Regimes 2, 5, 7, and 8. For each chemiluminescence experiment, six immunized rats were compared with six nonimmunized rats. Zymosan was opsonized with either immune or control serum, according to the cell group. Samples were set up in triplicate. As luminol may temporarily enhance neutrophil chemiluminescence by surface interactions with phagocytes (6), zymozan was not added to the cell/luminol mixtures until light output had subsided. When activity had diminished (within 5 min), zymosan was added and measurements were made of light output integrated during 2-s intervals at 2, 5, 10, 15, and 20 min.

ELISA Assay

Samples of serum and saliva were collected at the time of the clearance assay. Saliva flow was stimulated by intravenous administration of 20 mg/kg 2% (wt/vol) pilocarpine, and an average volume of 400 µl/rat was collected for 15 min. Blood was obtained by cardiac puncture and centrifuged at 500 × *g* to separate serum. One to two milliliters of serum per rat was obtained. Serum, saliva, and BAL were assayed for anti-NTHI-OMP specific antibodies of A, G, and M isotypes. Prior to the assay, BAL was freeze-dried, then reconstituted to 2 ml with distilled water. Serum and saliva were diluted in diluent buffer [2% (wt/vol) BSA in PBS/Tween 20 (0.05% wt/vol)] 1:100 and 1:4, respectively.

NTHI-OMP was prepared according to a modification of the technique of Barenkamp and coworkers (7) from a sonicate of whole NTHI (biotype I). Briefly, whole membrane fractions were harvested by ultracentrifugation from the bacterial sonicate and treated with 2% (wt/vol) sodium sarcosyl to dissolve inner membrane proteins. The detergent insoluble fraction (an outer membrane-rich preparation) was dissolved in 1% (wt/vol) deoxycholic acid by incubating at 45° C for 4 h. Protein concentration was measured by the method of Lowry and associates (8) and adjusted to 14 µg/ml.

The ELISA technique performed was a modification of that used by Yeung and colleagues (9). The method was optimized using anti-rat specific antibodies of IgA, IgG, and IgM isotypes. To correct for between plate variation, quality controls of a control serum (prepared by two intraperitoneal immunizations of NTHI/FCA, two weeks apart) were included on each plate, diluted 1:100. Sample optical density readings were adjusted according to the percentage deviation of the control serum value from the mean of all estimates for the control serum. The within assay coefficient of variation after correction was 4.3% for IgA, 3.7% for IgG, and 6.0% for IgM.

Statistical Analysis

For the pulmonary clearance assays, immunized groups were tested for difference from control groups by a two-tailed Student's *t* test. Control groups were pooled after an analysis of variance estimation demonstrated that they were derived from one population. BAL total cell counts were also compared by two-tailed Student's *t* tests. Variation between total cell count data from control groups was assessed by analysis of variance. For chemiluminescence results, *t*-tests com-

pared the mean value of immunized and nonimmunized curves. Values of $p < 0.05$ were considered significant (10).

Results

Clearance Assays

Assessment of optimal regimes. The results of bacterial clearance studies performed on differing inoculation regimes are summarized in table 1. Optimal results were obtained with regimes in which sequential stimulation of GALT and bronchus-associated lymphoid tissue (BALT) was undertaken. In Regime 1, a NTHI/FCA emulsion was administered by IPP injection before intratracheal boosting two weeks later. This regime resulted in marked acceleration of NTHI clearance in the 4-h period after intratracheal bacterial instillation. Regimes 2 and 3 demonstrated that FCA was an amplifying but not a vital factor, as in its absence, acceleration of bacterial clearance was demonstrable, and alone, no such effect occurred. Regime 9, which substituted two weeks of NTHI feeding for the IPP injection, also led to accelerated NTHI clearance. Other regimes, including local stimulation alone (Regimes 4 and 5) and systemic plus local stimulation (Regime 8), had no effect on the NTHI clearance rate. In regimes where GALT alone was stimulated, the NTHI clearance rate was retarded.

Specificity of action. Five rats were immunized according to Regime 2 and, on Day 21, were challenged with *Klebsiella pneumoniae*, an unrelated gram-negative respiratory pathogen. Mean numbers of *K. pneumoniae* in immunized rats were 6.46 ± 0.27 ($\log_{10}$), compared with 6.99 ± 0.55 ($\log_{10}$) in nonimmunized rats. There was thus no significant difference between immunized and nonimmunized rats with respect to *Klebsiella* clearance.

Duration of action. Groups of six rats were immunized with either NTHI or NTHI + FCA IPP and left for 40 and 60 days before intratracheal boosts and clearance assessment (Regimes 10 and 11). At 40 days, a significantly enhanced clearance rate was observed. The enhancement was equal in degree in the group given NTHI + FCA and the group given NTHI alone, indicating that any nonspecific adjuvant effect of FCA had worn off, and the specific effect provided by the NTHI remained. By 60 days, the protective value of the vaccination was lost, with no significant difference in rate of clearance between immunized and nonimmunized rats.

Behavior of Phagocytes

Yield of cells from bronchial lavage. Total cell counts from each of the four inoculation regimes are tabulated (table 2). At both 2 and 4 h, significantly greater numbers of cells were washed from the bronchi of rats immunized according to Regime 2, and Regimes 5 and 8 showed trends towards the acceleration of recruitment of cells. All cell populations were viable (> 85%) by trypan blue exclusion. Differences in control values at each time point were not significant ($p > 0.05$). Most of the cells recruited were neutrophils, with absolute numbers of macrophages changing very little. At 2 h, neutrophils comprised two-thirds of total cells in Regimes 2, 5, and 8, while only making up one-third of total cells in Regime 7. By 4 h, polymorphs comprised 90% of total cell count in all groups.

Cell activity. The only regime to exhibit significantly higher phagocytic activity in immunized as compared to control rats was Regime 2, where there was dual immunization of gastrointestinal and respiratory tracts (figure 1). Activity was higher at both 2 and 4 h after challenge. In other groups tested (Regimes 5, 7, and 8), no significant difference between immunized and control values was observed ($p > 0.05$).

Antibody Responses

The results of antibody assays on serum and secretions are summarized in table 3. No correlation could be recognized between the presence of raised levels of NTHI-specific antibody and the ability to accelerate pulmonary clearance of this organism.

Discussion

A quantitative model of clearance of NTHI from the respiratory tract of the rat determines both the efficacy of various immunization schedules and the mechanism of clearance of this nonencapsulated bacterium. It was demonstrated that both the GALT and the BALT required stimulation by antigen for significantly enhanced clearance and that the effect of oral priming was specific and persisted for about six weeks. No correlation could be drawn between enhanced clearance of NTHI and presence of specific antibody in local secretions or serum. The rate of recruitment of neutrophils and level of activation of phagocytes, however, was shown to be higher in animals immunized according to the successful regime, Regime 2.

TABLE 2

INTRABRONCHIAL CELL RESPONSE AFTER IMMUNIZATION

Regime	Bacterial Clearance Rate	Number in Group	Two Hours				Four Hours			
			Total Cell Count		% Polymorphs		Total Cell Count		% Polymorphs	
			Immunized	Control	Immunized	Control	Immunized	Control	Immunized	Control
2. NTHI IPP/NTHI IT	enhanced	7	$4.8 \times 10^{6*} \pm 0.6 \times 10^6$	$2.8 \times 10^6 \pm 0.4 \times 10^6$	65 ± 2	28 ± 2	$2.2 \times 10^{7**} \pm 0.3 \times 10^7$	$8.5 \times 10^6 \pm 1.2 \times 10^7$	95 ± 0.9	91 ± 1
5. NTHI IT/NTHI IT	no change	6	$5.8 \times 10^6 \pm 1.0 \times 10^6$	$3.1 \times 10^6 \pm 0.5 \times 10^6$	66 ± 3	27 ± 1	$2.3 \times 10^7 \pm 0.5 \times 10^7$	$1.9 \times 10^7 \pm 0.5 \times 10^7$	91 ± 2	89 ± 1
7. NTHI IPP/PBS IT	retarded	4	$2.6 \times 10^6 \pm 0.5 \times 10^6$	$2.3 \times 10^6 \pm 0.9 \times 10^6$	32 ± 7	31 ± 2	$9.7 \times 10^6 \pm 4.8 \times 10^6$	$1.6 \times 10^7 \pm 0.7 \times 10^7$	89 ± 1	90 ± 2
8. NTHI SC/NTHI IT	no change	6	$7.7 \times 10^6 \pm 1.9 \times 10^6$	$2.9 \times 10^6 \pm 0.6 \times 10^6$	59 ± 6	37 ± 3	$2.1 \times 10^7 \pm 0.4 \times 10^7$	$9.2 \times 10^6 \pm 0.6 \times 10^6$	86 ± 1	89 ± 3

Definition of abbreviations: NTHI = nontypable *Haemophilus influenzae*; IPP = intra Peyer's patch; IT = intratracheal; SC = subcutaneous.

Total cell counts (± standard error) and polymorph percentages at 2 and 4 h after intratracheal challenge with NTHI. Variation between control groups at each timepoint is nonsignificant ($p > 0.05$ in all comparisons). Within each regime, immunized and control groups are compared by two-tailed Student's *t* tests. *$p < 0.05$, **$p < 0.001$.

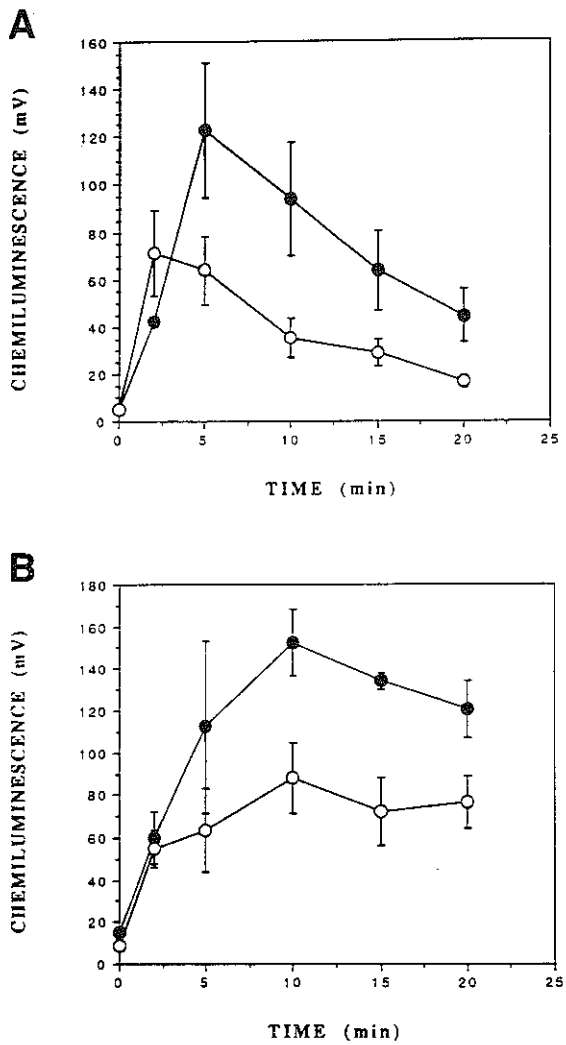

Fig. 1. Chemiluminescence (mean ± SE) of bronchoalveolar phagocytes at 2 h (A) and at 4 h (B) after intratracheal challenge with live nontypable *Haemophilus influenzae*. Rats were immunized according to Regime 2. Closed circles = immunized; open circles = control.

Demonstrations of a functionally interconnecting network of lymphoid tissues (11, 12) and the suggestion that an optimal local immune response in the lungs depends upon prior sensitization of GALT (13) led to the investigation of a rat model of bronchial immunization by a dual GALT-BALT immunization system. A functional assay of pulmonary bacterial clearance was chosen to measure immunity, instead of the evaluation of the presence of antibody or antibody-containing cells, which has been used in previous studies (14–16). Although lung homogenates are more commonly used in microbial clearance assays, bronchial washings were used in these experiments, as the NTHI in use is not an invasive strain in rats. Our studies indicated no increased bacterial yield in homogenates (unpublished data), an observation consistent with the findings of Libich and coworkers (16) in relation to *K. pneumonia* in mice. Bronchial washings are more conveniently collected and allow concurrent studies of bacteriology, cytology, and immunology.

Rodents are not natural hosts for most bacteria that are important respiratory pathogens in man. Often, specific interaction with the immune system does not take place, and bacteria may be handled as inert particles (17). Nontypable *H. influenzae* is not found naturally in rat lungs as either a pathogen or a commensal, and even at doses of 10^{12} cfu, live bacteria can be cleared from the lungs of nonimmunized rats within 48 h (unpublished data). In the current study, however, the marked enhancement of NTHI clearance achieved after mucosal immunization implies recruitment of an adaptive immune response.

In the current model, enhanced clearance required immunization of both GALT and BALT. The GALT could be immunized directly by injection into the Peyer's patches, with or without adjuvant, or by ingestion of killed NTHI. To enhance clearance rate, the initial immunization of GALT could not be replaced by either intrabronchial immunization

TABLE 3

CONCENTRATION OF SPECIFIC ANTIBODY IN SERUM AND SECRETIONS

Regime	Number	IgA			IgG			IgM			Bacterial Clearance Rate
		sal	ser	BAL	sal	ser	BAL	sal	ser	BAL	
Nonimmunized	38	274 ± 55	337 ± 36	234 ± 34	101 ± 17	693 ± 38	430 ± 38	41 ± 12	264 ± 27	115 ± 19	
1	6	n/s	n/s	n/s	n/s	n/s	n/s	n/s	650 ± 121	423 ± 60	accelerated
2	8	611 ± 61	n/s	88 ± 95	220 ± 46	961 ± 39	783 ± 65	n/s	611 ± 62	350 ± 39	accelerated
3	6	n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s	unchanged
4	8	n/s	n/s	n/s	24 ± 9	n/s	n/s	n/s	n/s	n/s	unchanged
5	6	n/s	628 ± 42	1,728 ± 320	n/s	929 ± 89	779 ± 115	n/s	n/s	358 ± 90	unchanged
6	6	n/s	n/s	n/s	n/s	n/s	n/s	n/s	455 ± 32	n/s	retarded
7	6	n/s	554 ± 63	741 ± 127	226 ± 47	984 ± 90	768 ± 63	10 ± 5	418 ± 53	257 ± 44	retarded
8	6	40 ± 20	545 ± 43	1,114 ± 148	n/s	n/s	784 ± 38	n/s	453 ± 36	433 ± 41	unchanged
9	6	n/s	n/s	n/s	n/s	n/s	n/s	n/s	n/s	250 ± 33	accelerated
10 (a)	5	n/s	n/s	n/s	n/s	855 ± 17	844 ± 66	n/s	627 ± 69	n/s	accelerated
10 (b)	5	n/s	n/s	1,316 ± 106	340 ± 25	876 ± 32	837 ± 35	84 ± 12	693 ± 79	456 ± 49	accelerated
11 (a)	7	n/s	728 ± 106	1,954 ± 46	641 ± 91	1,169 ± 8	643 ± 65	120 ± 19	524 ± 54	480 ± 11	unchanged
11 (b)	7	n/s	n/s	1,822 ± 178	401 ± 36	860 ± 32	1,054 ± 94	135 ± 21	n/s	402 ± 19	unchanged

Definition of abbreviations: sal = saliva; ser = serum; BAL = bronchoalveolar lavage; n/s = not significant.

Anti-NTHI antibody in secretions and serum as measured by enzyme linked immunosorbent assay with NTHI-OMP as base antigens. Results shown are those antibody levels significantly different from mean nonimmunized levels, as measured by Student's *t* test where $p < 0.05$ was considered significant.

or systemic immunization. These data strongly support the concept of a common mucosal system that involves a specific intermucosal cell traffic (18–20) and a requirement for antigen to be presented to the distant mucosal site to amplify response (21–23). In the ruminant at least, the dominant direction of this traffic is from the gut to the bronchus (13). The current study indicates that antigen within the bronchus not only activates relocated immune effector cells but is necessary for their retention within the lungs, as animals with immunization of GALT alone had a significant reduction of clearance rate. Examination of data obtained using a Sendai virus-murine system suggested a similar trend in animals orally immunized without subsequent intrabronchial antigen (15), although insufficient data was available for analysis.

The duration of action of the immunization process was at least 40 days, considerably longer than previously reported in other pulmonary clearance models, including systems involving local immunization with *Pseudomonas aeruginosa* (24), *K. pneumoniae* (16, 25), and *Serratia marcescens* (27) and systemic immunization with *H. influenzae* type b (27). This prolonged state of enhanced clearance reinforces the potential role of GALT in the development of immunity.

Enhanced clearance appeared to be specific as has been found in other bacterial clearance systems (24, 26–28). An assay of isotype-specific antibody to NTHI-OMP antigens in saliva, serum, and bronchial washings, however, failed to clearly associate enhanced clearance with any particular antibody profile. This contrasts with studies using encapsulated *H. influenzae* (27) that demonstrated enhanced pulmonary clearance of that organism in mice after systemic immunization or intravenous administration of a specific monoclonal IgG antibody. Recent studies by Hansen and associates (29) demonstrated enhanced pulmonary clearance of NTHI, after intraperitoneal immunization with live organisms, which appeared to correlate with antibody in serum and bronchial washings. Intraperitoneal immunization, however, is an effective method of presenting antigen to GALT (4), and thus with a concomitant activation of both systemic and mucosal immune systems, conclusions about precise mechanisms become difficult. Other model systems of enhanced pulmonary clearance have been described involving *K. pneumoniae* (16) and *Salmonella minnesota* (28) where no correlation between antibody concentrations and enhanced clearance could be demonstrated. Our negative observations do not exclude antibody as an integral part of the demonstrated immunity. It is possible that low assay sensitivity (e.g., for salivary IgA antibody) or the presence of a cytophilic antibody or an antibody of a unique specificity may account for the lack of correlation in our studies.

We therefore analyzed putative effector mechanisms by quantitating the recruitment of phagocytic cells within the bronchus after bacterial challenge. The neutrophil response was both more brisk and associated with higher levels of chemiluminescence activity when cells were tested from rats immunized by the dual route as compared with other immunization protocols. Sensitized T lymphocytes purified from thoracic duct lymph can replace gut immunization in this model (unpublished observations). As significant amounts of antibody in bronchoalveolar washings and serum can be induced by immunization by a variety of regimes not associated with enhanced clearance, T cell mediated neutrophil chemotaxis and activation become candidate mechanisms for the enhanced clearance observed after priming of GALT.

Despite the limitations of the NTHI/rat clearance system as a model for acute bronchitis in man, several comments can be made. First, the model further consolidates the concept of a common mucosal system being activated through oral immunization to modulate a colonizing process of the bronchus mucosa. Second, it parallels the human disease in that antibody concentrations in serum and secretions do not appear to correlate with protection (2). Third, the immunization data emphasize the importance of dual gut-bronchus immunization. It is probable that in the human disease, colonization itself provides sufficient stimulation of BALT in subjects with damaged airways, as most are heavily colonized with NTHI (1). Finally, it identifies enhanced neutrophil chemotaxis and cell activation as a possible therapeutic target for control of bacterial colonization in subjects prone to recurrent acute bronchitis.

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