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A role for intestinal T lymphocytes in bronchus mucosal immunity

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SUMMARY

Rats immunized by intra-Peyer's patch (IPP) injection with non-typable *Haemophilus influenzae* (NTHI) have been shown to clear this organism from the respiratory tract faster than non-immunized rats. We therefore performed a series of experiments in order to determine the mechanism of action of the enhanced pulmonary clearance. The experiments show that homing of intestinal T cells to the respiratory tract is an important component in the observed immunity, while specific antibody adsorbed to bacteria does not influence pulmonary bacterial clearance rate. Mucosally derived lymphocytes were collected from the thoracic duct of rats primed by IPP inoculation with NTHI, and intravenously transfused to recipient rats. These rats were shown to clear bacteria from bronchial spaces faster than non-transfused rats, or rats transfused with non-immune lymphocytes. Lymphocytes collected from the spleens of immunized rats were also capable of conferring the ability to accelerate pulmonary clearance. When thoracic duct lymphocytes (TDL) purified for T lymphocytes were transferred to recipients, the NTHI clearance rate was accelerated. In experiments to evaluate the activity of specific antibody, it was demonstrated that NTHI opsonized with antibody from bronchial washings of immunized rats was not cleared from the respiratory tract of naive rats faster than non-opsonized controls. These data indicate that immune clearance of NTHI from the respiratory tract following gut immunization is dependent upon antigen-primed lymphocytes, that primed T cells are capable of conferring this protection, and that a primary role for specific antibody in the process cannot be established.

INTRODUCTION

Non-typable *Haemophilus influenzae* (NTHI) has been implicated as a major factor in the aetiology of acute bronchitis in subjects with chronic bronchitis.[1-3] The success of an orally presented killed bacterial vaccine in reducing the number of acute attacks of bronchitis[4] has suggested the importance of activated gut-associated lymphoid tissue (GALT) in the induction of distant mucosal immunity in man. However, the mechanism of protection has not been defined.

The role of antibody in protection against NTHI respiratory infections has been questioned in both human and rat models. High levels of NTHI-directed antibody have been demonstrated in serum, saliva and sputum of patients with chronic bronchitis[5,6] and in one study the level of specific antibody in these body fluids directly correlated with the incidence of infection and mortality observed in these patients.[6] This is despite the fact that bactericidal antibody to NTHI has been demonstrated and shown to be protective in NTHI-related otitis media.[7-9] Musher *et al.*[2] have shown that chronic bronchitic patients can support high colonization rates of NTHI in the presence of significant levels of bactericidal and opsonic activity in the serum, and have suggested[10] that IgA in bronchopulmonary secretions blocks the bactericidal and opsonizing effects of antibody to NTHI.

A rodent model of NTHI immunization showed that intestinally immunized rats could clear NTHI from lungs faster than non-immunized rats only when immunization involved sensitization of gut-associated lymphoid tissue followed by sensitization of bronchus-associated lymphoid tissue. The presence of high levels of antibody in serum and bronchial washings specific for NTHI outer membrane protein was shown not to guarantee protection.[11] A model of Sendai virus immunization in mice[12] also demonstrated the need for dual gut-bronchus immunization for protection to occur.

The object of the present study was to assess the cellular and humoral basis of enhanced respiratory immunity in intestinally immunized rats.

MATERIALS AND METHODS

Rats

Male inbred DA rats were kept in specific pathogen-free

Abbreviations; CFA, complete Freund's adjuvant; FCS, foetal calf serum; GALT, gut-associated lymphoid tissue; IPP, intra-Peyer's patch; NTHI, non-typable *Haemophilus influenzae*; PBS, phosphate-buffered saline; PP, Peyer's patch; TDL, thoracic duct lymphocytes.

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conditions until the commencement of the immunization protocol. All were aged 6–8 weeks at the beginning of each experiment.

Donor rats

Formalin-killed NTHI was prepared as described previously.[11] Immunized donor rats were given intra-Peyer's patch (IPP) antigen as described by Husband & Dunkley.[13] NTHI, prepared to a concentration of 2×10^{10} colony-forming units (CFU)/ml, was emulsified in an equal volume of complete Freund's adjuvant (CFA), and distributed via a 27 g needle in 2–5 μl volumes subserosally adjacent to each Peyer's patch (PP). Immunization of all the PP in the small intestine constituted a total dosage of approximately 5×10^8 CFU NTHI per rat. Non-immunized donor rats were not injected. Five days after immunization, thoracic duct cannulations were performed, as described by Bollman, Cain & Grindlay.[14] Lymph was collected in sterile flasks containing 5 ml phosphate-buffered saline (pH 7·3) (PBS), to which had been added 20 U/ml heparin. The lymph was harvested each day for 4 days and the sterile collection flask containing PBS/heparin was replaced daily. In experiments in which spleen cells were transferred, donor rats were not cannulated.

Recipient rats

Recipient rats received 4 daily doses of lymphocytes via an intravenous cannula. On Days 1 and 4, they also received intratracheal doses of NTHI, prepared to a concentration of 1×10^{10} CFU/ml. This was instilled in a 50-μl volume into the lungs via a 20 g catheter placed intratracheally. On Day 7, assessment of clearance rate was carried out as described previously.[11] Briefly, a standardized inoculum of NTHI from an overnight culture was instilled into the lungs via the trachea. Four hours later, bronchial washings were collected by five sequential 2-ml lavages of the lungs with warmed isotonic saline containing 100 U/ml heparin, so that a final volume of 10 ml was retrieved. The lavage fluid was serially diluted and plated onto chocolate agar. Numbers of CFU counted provided an indication of the number of bacteria remaining within the lungs.

Control rats either received non-immune lymphocytes or were not transfused. The intratracheal NTHI was administered to all control rats on Days 1 and 4 in parallel with test rats.

Preparation of cell suspensions

TDL collected from donor rats were washed twice in PBS containing calcium and magnesium and supplemented with 5% (v/v) foetal calf serum (FCS). Viability was assessed by trypan blue exclusion, and in all cases was greater than 97%. For adoptive transfers, the cells were resuspended in PBS. In experiments where B-cell depleted TDL were required, cells were passed over nylon-wool columns[15] before panning on anti-immunoglobulin-coated dishes using a modification of the technique of Wysocki & Sato.[16] Plastic Petri dishes (6 cm diameter; Lab-Tek, Miles Laboratories, Australia) were coated by overnight incubation at 4° with rabbit anti-rat IgG (Dakopatts, Glostrup, Denmark) diluted 1/50 in borate buffer (pH 8·7). The next day plates were rinsed once with PBS and then three times with PBS containing 1% (v/v) FCS. Cells to be panned were resuspended to 15×10^6 cells/ml in PBS. A 2-ml volume of this suspension was added to each of the plates, which were then incubated for 90 min at 4° with gentle swirling every 30 min, after which the non-adherent cells were gently aspirated with the supernatant. Aliquots of the resulting cell populations were characterized by flow cytometry after staining with the monoclonal antibodies OX12 (specific for rat Fab_2 fragments) and OX19 (specific for rat T lymphocyte glycoprotein) (Sera-Lab, Crawley Down, Sussex, U.K.) and a secondary FITC-conjugated anti-mouse antibody (Caltag Laboratories, San Francisco, CA).

Spleens were removed aseptically from donor rats. They were then chopped into small pieces and passed gently through a fine wire mesh into sterile PBS/FCS. This suspension was left to stand for 10 min to allow clumps and debris to settle. The cell suspension was centrifuged and the cells resuspended in PBS before adoptive transfer.

Opsonization/challenge studies

Bronchial washings were collected (see above) from rats immunized according to the optimal regime (see above) or from non-immunized rats. The fluid obtained was centrifuged at 200 *g* to remove cells and incubated at 56° to inactivate complement. NTHI was grown overnight on chocolate agar at 37° in a 5% CO_2 incubator and resuspended to a final volume of 1×10^{10} CFU/ml in PBS. Two 1-ml aliquots were pelleted in sterile microfuge tubes and resuspended in either immunized or non-immunized bronchial washing fluid. A third aliquot was resuspended in PBS. The suspensions were incubated for 1 hr at 37°, then repelleted. The bronchial washing fluid supernatants were removed and stored for analysis. The bacterial pellets were resuspended in 1 ml PBS and administered intratracheally to naive rats. Four hours later rats were killed and bronchial washings collected. Numbers of NTHI CFU were then quantified. Bronchial washing fluid both pre- and post-incubation with NTHI was subsequently assayed by an ELISA technique to quantify the amount of antibody adsorbed to the bacteria. The ELISA technique employed was a modification of the technique of Wallace *et al.*[11] Briefly, the major modifications were: (i) for each sample to be tested, duplicate wells were coated both with and without NTHI antigen. The optical densities of the wells with no antigen were then subtracted from the optical densities of the wells coated with NTHI antigen, thus eliminating variation caused by non-specific binding of samples to the plates. (ii) The plates included a standard curve of hyperimmune serum[11] from which ELISA units were calculated according to the following method: the lowest hyperimmune serum dilution was given an arbitrary value of 10, and the highest dilution an arbitrary value of 1. A linear graph of ELISA unit value χ optical density was constructed from the standard curve, and sample ELISA unit values were then calculated by interpolation.

Statistical analysis

Groups of rats within each experiment were compared by a Mann–Whitney test for non-parametric data. P values <0.05 were considered significant.

RESULTS

Whole TDL transfer

Recipient rats were infused on four consecutive days with whole washed syngeneic TDL populations. Rats received, on average, $2\text{–}3 \times 10^8$ cells per day collected from the pooled yield of an

Table 1. Pulmonary clearance assay on transfused rats

Exp.	Transfused rats (CFU; mean ± SE)	Controls rats (CFU; mean ± SE)	% of control	*P* value
1. Rats receiving whole populations of TDL ($2–3 \times 10^8$ cells/day) ($n = 7$)	$1{\cdot}4 \times 10^5 \pm 0{\cdot}5 \times 10^5$	(a) $6{\cdot}3 \times 10^6 \pm 0{\cdot}9 \times 10^6$ (b) $2{\cdot}9 \times 10^6 \pm 0{\cdot}4 \times 10^6$	(a) 2·0 (b) 5·0	<0·05 <0·05
2. Rats receiving reduced numbers of TDL ($4–5 \times 10^7$ cells/day) ($n = 5$)	$3{\cdot}7 \times 10^4 \pm 1{\cdot}9 \times 10^4$	$1{\cdot}3 \times 10^5 \pm 0{\cdot}6 \times 10^5$	29·0	>0·05
3. Rats receiving purified T cells ($8–9 \times 10^7$ cells/day) ($n = 4$)	$1{\cdot}1 \times 10^4 \pm 8{\cdot}7 \times 10^4$	$1{\cdot}4 \times 10^6 \pm 0{\cdot}4 \times 10^6$	0·8	<0·05
4. Rats receiving splenic cells ($2–3 \times 10^8$ cells/day) ($n = 4$)	$3{\cdot}7 \times 10^4 \pm 1{\cdot}2 \times 10^4$	$5{\cdot}8 \times 10^5 \pm 2{\cdot}2 \times 10^5$	6·4	<0·05

Rats received lymphocyte transfusions for 4 consecutive days, with intratracheal NTHI inoculation on Days 1 and 4 and clearance rate assessment on Day 7. All control groups except (a) of Exp. 1 received intratracheal boosts but no lymphocytes. Group (a) of Exp. 1 received in addition $2–3 \times 10^8$ non-immune cells per day over the transfusion period. Data are presented as the mean ± standard error of observations from the number of rats indicated.

Table 2. Opsonization/challenge studies

Group	No. of CFU (±SE) remaining in BAL 4 hr after challenge
NTHI + immunized BAL	5·92 ± 0·09
NTHI + non-immunized BAL	5·63 ± 0·18
NTHI (not opsonized)	6·02 ± 0·24

Bronchial washings, collected from immunized and non-immunized rats, were centrifuged to remove cells and heat treated to inactivate complement. Bacteria were incubated with either immunized or non-immunized bronchial washings and administered intratracheally to naive rats. Rate of pulmonary bacterial clearance was measured (see text). Results are expressed as $\log_{10}$ figures.

NTHI, non-typable *Haemophilus influenzae*; BAL, bronchial washing fluid.

Table 3. Depletion of antibody from immunized BAL following incubation with NTHI

	ELISA values before and after incubation with NTHI	
	Pre-incubation	Post-incubation
IgA	2·16 ± 0·2*	0·33 ± 0·04
IgG	0·99 ± 0·1	0·21 ± 0·01
IgM	0·99 ± 0·2	<0·05

NTHI was incubated with immunized bronchial washing fluid, and 'before' and 'after' samples were tested in an ELISA to quantitate the extent of depletion of antibody.

NTHI, non-typable *Haemophilus influenzae*; BAL, bronchial washing fluid.

* Results are expressed in ELISA units and represent the arithmetic means of four samples run in triplicate.

equivalent number of immunized donors. The cells were pooled to optimize numbers of cells given to each recipient, allowing for the variation in yield between different donors over different days. Rats receiving immune cells were compared with rats receiving non-immune cells and also with non-transfused rats with respect to their ability to clear NTHI from the respiratory tract. Rats receiving immune cells had significantly fewer (2%) bacteria remaining in their respiratory tracts than rats receiving non-immune cells and non-transfused rats ($P < 0{\cdot}05$) (Table 1). Because non-transfused rats and rats receiving non-immune lymphocytes cleared NTHI at the same rate, the former treatment was used as a control in all subsequent experiments.

Purified T-cell transfer

Purified T cells were prepared from TDL by B-cell depletion. The purified T-cell populations consistently contained 95% $OX19^+$ cells, and less than 5% $OX12^+$ cells. Animals receiving these cells (in numbers of $8–9 \times 10^7$ per rat) were able to demonstrate significantly improved pulmonary bacterial clearance ($P < 0{\cdot}05$), of the same magnitude as those receiving whole populations of unfractionated lymphocytes (Table 1). That is, the number of bacteria remaining in the lungs 4 hr after bacterial instillation in transfused rats was 0·8% of the number remaining in control rats. To check whether residual B cells were responsible for clearance enhancement, recipient rats were infused with $4–5 \times 10^7$ immune TDL per day, and compared to a group of non-transfused rats. In this case, the number of B cells transfused (which would equate to about 40% of total numbers,

that is $1\cdot6\times10^7$ cells) then exceeded the numbers that would have been transfused in the purified T-cell transfer (at most 5% of 9×10^7, or $4\cdot5\times10^6$) by over threefold. Each group received the two intratracheal immunizations in the transfusion period. Rats receiving immune cells did not show significantly improved clearance of NTHI ($P>0\cdot05$) (Table 1).

Systemic (spleen) cell transfer

Recipient rats were infused with washed spleen cell populations, receiving $2–3\times10^8$ cells per day. When compared with non-transfused rats, these rats demonstrated significantly accelerated clearance of NTHI ($P<0\cdot05$), of the same magnitude as rats receiving whole unfractionated TDL (Table 1) (transfused rats had 5% of the number of bacteria remaining in the lungs).

Opsonization/challenge studies

Table 2 illustrates the results of the opsonization/challenge studies. Naive rats challenged with NTHI opsonized with bronchial washing fluid harvested from immunized rats were unable to clear these bacteria faster than rats challenged with non-opsonized bacteria, or with bacteria opsonized with bronchial washing fluid harvested from non-immunized rats. The amount of antibody absorbed from each sample was quantified. At least 80% of the antibody in bronchial washing fluid was removed by one adsorption step (Table 3).

DISCUSSION

This series of experiments indicates that antigen-primed gut-associated T lymphocytes activated following immunization of Peyer's patches are able to contribute to functional immunity in the respiratory tract. This has particular relevance in the understanding of immunity against NTHI respiratory infections, in the light of accumulating evidence which questions the role of specific antibody in protection against this organism.

Accelerated bacterial clearance from the respiratory tract following immunization of intestinal lymphoid tissue provides functional support for the existence of a common mucosal system, where immune lymphocytes migrate from one mucosal site to another.[17] Weisz-Carrington *et al.*[18] showed that oral presentation of antigen resulted in the appearance of antigen-specific IgA plasma cells in the mucosae of the gut and lungs as well as other mucosal sites, while Pierce & Cray[19] confirmed that following intestinal sensitization, or adoptive transfer of sensitized TDL, specific antibody-containing cells appeared in the gut and the trachea. An enhanced antibody-containing cell response which could be abrogated by chronic drainage of the intestinal lymph has also been noted in the upper respiratory tract of sheep following dual intraperitoneal–intratracheal immunization.[20] Following feeding of alloantigens to rats, specifically reactive T cells could be demonstrated in lungs.[21] Dunkley & Husband[22] demonstrated the presence of IgA-specific T-helper cell populations in thoracic duct lymph following IPP immunization with a soluble antigen. However, a functional contribution of intestinal T cells to immunity remains to be demonstrated.

An orally administered killed NTHI vaccine which reduces the incidence of acute bronchitis in man does not correlate with specific antibody levels in local secretions.[4] Musher *et al.* have noted that colonization and infection with NTHI appear to occur despite the presence of bactericidal and opsonic antibody in the serum of both healthy subjects[1] and those hospitalized for acute lower respiratory tract NTHI infection.[2] They suggest[10] that in man secretory IgA in bronchial washings blocks bactericidal and opsonizing activity of serum IgG. A rat model established in this laboratory provides data consistent with these suggestions.[11] In this model, high levels of specific antibody occur in bronchial washings of rats immunized according to a regime which enhances the rate of NTHI pulmonary clearance, and also in rats immunized by alternative regimes which do not confer protection. It is possible that the assay system used in this laboratory does not highlight the presence of a vital antibody (or antibody combination) responsible for conferring protection, thus indicating only an apparent lack of correlation between antibody levels and protection. However, the failure of opsonized bacteria to be cleared at a greater rate from the respiratory tract argues against this being the case.

The role of antibody in protection against NTHI colonization of the respiratory tract remains obscure. The above experiments are further supportive evidence that the presence of specific antibody in serum and bronchial washings may be incidental to immune protection A recent study in which Hansen *et al.*[23] correlated specific antibody in bronchial washings with enhanced pulmonary clearance of NTHI following intraperitoneal immunization with live organisms may have observed a coincidental occurrence, as alternative regimes were not examined. Alternatively, antibody may be important in immunity to NTHI in the rat model in conjunction with cellular factors.

In the current study, the cellular basis of rat pulmonary immunity to NTHI has been investigated. The data indicate that immunity arises from the action of migrating intestinal lymphocytes. T cells were crucial to this process. Both unfractionated and purified T-cell populations of TDL were capable of enhancing the pulmonary clearance rate. Other experiments were conducted to assess the contribution of B cells and humoral antibody in pulmonary immunity to NTHI. One approach, to adoptively transfer T-depleted TDL, was abandoned following technical difficulties in creating a sufficiently pure population of B cells. Also, following pilot experiments with irradiated rats, it was found that an intact recipient lymphocyte repertoire was necessary for specific antibody production to occur following immunized lymphocyte transfer. It was concluded that transfer of purified B-cell populations to non-irradiated rats would therefore not be capable of generating a significant antibody response in recipients. Instead, an alternative approach to assess B-cell contribution to immunity was employed, in which fresh inocula of NTHI were opsonized with specific antibody derived from the bronchial washings of immunized rats. Opsonized bacteria were not cleared from the respiratory tract faster than non-opsonized bacteria, indicating either that insufficient antibody was adsorbed to the bacteria, or that the antibody was not opsonic. It would appear, however, that antibody binding sites on the bacteria were saturated following incubation with immunized bronchial washings, as significant levels of antibody in bronchial washings remained following incubation. Further assessment of the opsonic activity of anti-NTHI antibody by *in vitro* assays would be fruitful.

To establish whether or not antibody was functional in enhancing the clearance rate of transfused rats, specific anti-

body titres were measured in recipients. Both infused and non-infused rats had significant levels of specific antibody in bronchial washings, with no significant difference between the groups (data not shown). Clearly, intratracheal boosting was responsible for local antibody production in the non-infused (and perhaps also the infused) rats. This shows that the presence of specific antibody alone does not guarantee protection, which is further support for our proposition that transferred T cells are providing an additional signal important to enhanced clearance rate.

The exact mechanism of action of intestinal T cells requires clarification. There are three possibilities. First, T cells could act to 'help' B cells in the production of specific antibody to NTHI. Although most available data indicate that antibody in bronchial secretions may not be relevant to immune clearance of this organism, antibody may contribute in conjunction with other effector functions. High levels of specific antibody were present in bronchial washings of rats receiving transfusions of immunized cells, but they were no higher than in bronchial washings of control rats which had received only the intratracheal boost. Second, lymphokine release could induce changes in the behaviour of lung phagocytes, and rapid recruitment of activated neutrophils has been demonstrated following gastrointestinal immunization.[11] Recent stuidies[24,25] have indicated that the T-cell-derived lymphokine TNF-β modulates neutrophil function by inhibition of locomotion and stimulation of respiratory burst. Third, T cells could be directly cytotoxic, a phenomenon which has been described *in vitro* in relation to another Gram-negative respiratory pathogen, *Pseudomonas aeruginosa*.[26]

In previous studies[11] an intratracheal boost in conjunction with gastrointestinal immunization was shown to be necessary for development of protective immunity in the respiratory tract. The importance of the presence of antigen at a distant mucosal site to retain intestinally derived specific immune cells, and to enhance their migration and proliferation at that site, has been stressed in other experimental systems with respect to both B cells[27-29] and T cells.[30] Therefore antigen was administered intratracheally to both test and control animals. Intratracheal immunization alone does not influence the rate of clearance of NTHI from the respiratory tract in this model.[11]

It could be argued that the use of CFA in this model could cause local inflammation at the site of the PP, and change the number and proportions of cells migrating from this site. However, following immunization, TDL populations remained almost exclusively lymphocytes, and both the absolute numbers and the ratio of B:T lymphocytes remained unchanged. Further, it has been demonstrated that immunization with CFA alone does not influence the rate of clearance of NTHI from the lungs,[11] which argues against the proposition that non-specifically activated T cells are stimulated to migrate in TDL following this procedure.

The variability in yield of NTHI from the lungs of challenged rats between experiments remains a consistent event in this assay system, despite attempts to standardize the procedures. Factors causing variability have been ascribed to environmental temperature and humidity on the day of the experiment, causing variable viability of NTHI *in vitro*, and perhaps variation in efficacy of *in vivo* clearance mechanisms. However, despite this variability, within each experiment reported statistical measures have established significant differences between treated and untreated rats, and the experiments are reproducible in this sense, if not in the demonstration of equivalent absolute numbers of NTHI organisms retrieved from the lungs.

Thoracic duct cannulation provides the opportunity to compartmentalize the rat immune system into mucosal and systemic components. In experiments described above, the thoracic duct was cannulated at a point just above the cysterna chyli, where the overwhelming majority of lymphocytes are of gastrointestinal origin.[31] These data therefore support the concept of a mucosally restricted pattern of distribution of intestinally derived T cells consistent with the mucosal restriction of intestinal helper T lymphocytes previously demonstrated.[30] That cells derived from the spleen conferred the ability to enhance clearance could reflect either dissemination of antigen from the injection site with subsequent systemic priming, or the seeding of mucosal effector cells in the spleen. It is relevant that rats immunized systemically (subcutaneously) have been shown to be incapable of clearance acceleration.[11]

These studies show that intestinally derived antigen-activated lymphocytes are capable of conferring protection to NTHI at a distant mucosal site. The implication that immune clearance of bacteria from the bronchial lumen is mediated by T lymphocytes suggests a major role for activated T lymphocytes in the control of luminal phagocytosis of bacteria, an event previously linked more closely with opsonization due to antibody.

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