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In vitro and *in vivo* T cell responses in mice during bronchopulmonary infection with mucoid *Pseudomonas aeruginosa*

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SUMMARY

In vitro and *in vivo* T cell responses were determined during the course of bronchopulmonary infection with mucoid *Pseudomonas aeruginosa*. T cell responses were compared in two inbred mouse strains, namely BALB/c mice, which are resistant to the establishment of chronic bronchopulmonary *Ps. aeruginosa* infection, and C57Bl/6 mice, which have high numbers of bacteria in the lungs through 14 days post-infection. Unseparated lung cells and lung T cells from BALB/c mice exhibited significantly higher *in vitro* proliferative responses to both heat-killed *Ps. aeruginosa* and concanavalin A (Con A) than cells from C57Bl/6 mice through 20 days post-intratracheal infection with 10^4 colony-forming units (CFU) *Ps. aeruginosa*. Proliferation of unseparated lung cells but not lung T cells from BALB/c mice infected 6 days previously with 10^5 CFU *Ps. aeruginosa* was suppressed in response to Con A; these cells were unresponsive to specific antigen. Suppression of lymphocyte proliferation in the lungs of C57Bl/6 mice infected with 10^4 CFU *Ps. aeruginosa* and in BALB/c mice infected with 10^5 CFU was found to be mediated by adherent lung cells via the production of nitric oxide and prostaglandins. Determination of *in vivo* T cell-mediated responses in infected mice demonstrated that resistant BALB/c mice had high DTH and low *Pseudomonas*-specific antibody responses, while C57Bl/6 mice had low DTH and high antibody levels, in particular, IgG2b and IgM.

Keywords T cells *Pseudomonas aeruginosa* mice cystic fibrosis

INTRODUCTION

Individuals with cystic fibrosis (CF) are unusually susceptible to bronchopulmonary infection with *Pseudomonas aeruginosa*. The infections, caused by the more virulent, mucoid strains of the bacteria, are chronic and often fatal [1–3]. Although there is also a high incidence of *Pseudomonas* infection among burn patients and immunosuppressed individuals, infection in CF patients, even in the terminal stages, is localized to the lung [4]. Interestingly, the severity of pulmonary disease due to *Ps. aeruginosa* infection may vary from one patient to another even though they carry the same CFTR mutation, suggesting that another host gene(s) may regulate resistance to infection with this bacterium.

Investigation of the immune mechanisms(s) leading to host resistance to bronchopulmonary *Ps. aeruginosa* infection has focused primarily on the roles of antibody, complement and non-specific inflammatory responses [4,5]. In contrast, the role of cell-mediated immunity, in particular the role of T cells, has received little attention. Sorensen *et al.* [6–9] observed that peripheral blood lymphocytes from CF patients with advanced disease had impaired proliferative responses to *Pseudomonas* antigen *in vitro*. However, it is unclear if the defect in proliferation is intrinsic to T cells of CF patients *per se*, or if the function of these cells is depressed as a result of chronic *Pseudomonas* infection. In addition, it is unclear if the responsiveness of peripheral blood lymphocytes correlates with that of the local, interstitial T cells in the lung.

One of the problems in studying the role of lung T cells in the development of specific immunity to bronchopulmonary *Pseudomonas* infection has been the lack of a suitable animal model, since such studies would be impossible to perform in CF patients. Chronic proliferation of *Ps. aeruginosa* in the lung, with histologic damage similar to that observed in the lungs of CF patients, has been achieved in rats, guinea pigs, cats and, more recently, mice by tracheal inoculation of bacteria-impregnated agar beads to initiate infection [10–14]. The availability of genetically defined inbred strains, of specific immunological

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reagents and molecular probes as well as the recent availability of cystic fibrosis transmembrane conductance regulator (CFTR) gene knock-out animals [15,16] make the mouse an ideal host for the study of the role of T cells in immunity to bronchopulmonary *Ps. aeruginosa* infection.

We have established a mouse model of chronic bronchopulmonary *Pseudomonas* infection in our laboratory and have observed differences in the level of resistance among inbred mouse strains (Stevenson *et al.*, manuscript in preparation). Following intratracheal infection with 10^4–10^5 mucoid *Ps. aeruginosa* enmeshed in agar beads, BALB/c mice were found to clear the infection. Although the lungs of these animals were slightly congested, the general architecture of the lung was intact. Thus, this strain is resistant to the establishment of chronic bronchopulmonary *Pseudomonas* infection. C57Bl/6 mice, on the other hand, were found to be susceptible. These animals had high numbers of bacteria in the lung through 14 days post-infection, and marked congestion of the airways, hyperplasia of parenchymal cells, accumulation of inflammatory cells and microcolonies of bacteria attached to agar beads. In the present study, we compared the *in vitro* proliferative responses of interstitial lung T cells from normal and infected BALB/c and C57Bl/6 mice to specific antigen and the mitogen, concanavalin A (Con A). We also compared the *in vivo* immune responses, measured by DTH and *Pseudomonas*-specific antibodies in the serum, of these two mouse strains during infection.

MATERIALS AND METHODS

Mice

Age- and sex-matched BALB/c and C57Bl/6 mice, 6–10 weeks old, bred in our animal facility from breeding pairs obtained from Harlan Sprague Dawley (Indianapolis, IN) or purchased from Charles River (St Constant, Quebec, Canada) were used in all experiments. Food and water were provided *ad libitum*.

Pseudomonas aeruginosa

Pseudomonas aeruginosa, strain 508, was kindly provided by Dr Jacqueline Lagacé (Université de Montréal, Montréal, Québec, Canada). This strain has a mucoid appearance when grown on blood agar, and was originally isolated from the sputum of a cystic fibrosis patient at Ste-Justine's Hospital, Montreal, Canada.

Intratracheal infection

Bacteria were grown for approximately 18 h to log phase in 4% proteose peptone (Difco, Detroit, MI). A suspension of bacteria-impregnated beads was prepared using a modification of previously described methods [10,13,14]. Briefly, the bacterial suspension was concentrated 10-fold and 1 ml was added to 9 ml 1·5% trypticase soy agar (Difco). This mixture was added to 150 ml heavy mineral oil at 52°C while stirring rapidly with a magnetic stirring bar. Stirring was continued for 6 min at 22°C, followed by cooling with continuous stirring for 10 min more. The oil–agar mixture was centrifuged at 15000 *g* for 20 min to sediment the beads. The oil was removed and the beads subsequently washed three times in PBS at 400 *g* for 10 min at 22°C. The size of the beads was verified microscopically and only those preparations containing predominantly 100–150 μm size beads were used. The number of bacteria was estimated by homogenizing the bacteria-bead suspension using a Polytron Homogenizor (Brinkmann Instruments, Inc., Westbury, NY) and plating 10-fold serial dilutions on trypticase soy agar (BBL, Becton Dickinson, Cockeysville, MD). The plates were incubated overnight and the number of colony-forming units (CFU) counted. The number of bacteria enmeshed in the agar beads was usually 2×10^7 CFU/ml beads.

Mice were anaesthetized intramuscularly with 0·1 ml of a combination of ketamine hydrochloride (15 mg/ml) and xylazine (2 mg/ml). The trachea was visualized directly by a transverse cervical incision, intubated with a sterile, flexible 22 G cannula attached to a 1·0-ml syringe and 50 μl of the bacteria-bead suspension were inoculated into either lung. Mice were infected intratracheally with approximately 10^4–10^5 CFU. After inoculation, the incision was closed by suture. No animals developed wound infections and healing occurred in 2–3 days.

Lung cell suspensions

Mice were killed by overdose of xylazine and the animals exsanguinated by cutting the vena cava. The blood vessels were washed by infusion via the retro-orbital venous plexus with 7 ml Mg^{2+} and Ca^{2+}-free Hanks' balanced saline solution (HBSS; Gibco-BRL, Burlington, Ontario, Canada) containing 0·02% EDTA. Bronchoalveolar lavage (BAL) cells were removed by washing via cannulated trachea with 10 ml of HBSS with subsequent aspiration of liquid, repeated eight times. After these procedures, resulting in washing away free blood and BAL cells from the tissue, the lungs were removed from the thorax, washed twice with Medium L-15 (Gibco) supplemented with 2% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, UT), 20 mM HEPES (Flow Labs, Inc., Mississauga, Ontario, Canada) and 0·12% gentamycin and sliced into pieces 1–2 mm. Lung cell suspensions were prepared using a previously described modification of methods described by Holt *et al.* [17]. Briefly, lung slices were incubated with shaking for 1·5 h at 37°C in 20 ml of complete Medium L-15 with 2 mg/ml collagenase (Sigma Chemical Co., St Louis, MO), 50 μg/ml DNAase (Sigma) and 10 U/ml elastase (Boehringer-Mannhein, Laval, Quebec, Canada). A single-cell suspension was obtained after repeated pipetting followed by passage through a 20 G needle followed by a 25 G needle. The cells were washed three times with complete Medium L-15 and resuspended in complete tissue culture medium consisting of RPMI 1640 supplemented with 5% FCS, 25 mM HEPES and 0·12% gentamycin. The viability of the cells as determined by trypan blue exclusion was 80–90%.

Lung cell suspensions enriched for T cells were obtained as described previously [18]. Briefly, plastic adherent cells were removed by incubation of cell suspensions in culture media on 90 mm tissue culture dishes ($2{\cdot}5$–3×10^7 cells per dish) for 2 h at 37°C in 5% CO_2 atmosphere. Cell populations were enriched for T cells by passage of 5×10^7 plastic non-adherent cells through a 10-ml syringe column containing 0·7 g of nylon wool (Polyscience, Warrington, PA). The column was incubated for 1 h at 37°C and T cells eluted with warmed medium. The cells were washed and viability determined by trypan blue exclusion.

Spleen cell preparation

Spleens were aseptically removed and perfused with 10 ml of complete RPMI 1640. Cell suspensions were centrifuged at

350 *g* for 10 min. Erythrocytes were lysed with cold NH_4Cl (0·17 M) and the cells were washed twice in fresh medium. Membrane debris was removed by filtering the cell suspension through sterile gauze. Viability was determined by trypan blue exclusion and was always greater than 90%.

Proliferative responses

For proliferation assays, non-enriched lung cells were resuspended to 2×10^6 cells/ml and 50-μl aliquots were added to 96-well plates (Flow). Lung cells enriched for T cells were resuspended to 2×10^6 cells/ml. Aliquots of 50 μl were added to 96-well plates containing 3×10^5 syngeneic, antigen-presenting cells (APC) which consisted of spleen cells prepared, as described above, from normal animals and treated with mitomycin C (30 μg/ml) for 30 min at 37°C. Aliquots of 100 μl of spleen cells resuspended to $2{\cdot}5 \times 10^6$/ml were also added to 96-well plates. Cells were stimulated by the addition of 50 μl of Con A (2·0 μg/ml), heat-killed *Ps. aeruginosa* antigen (4×10^7 CFU/ml) or medium as control. Cultures were incubated for 48 h at 37°C, pulsed for 16 h with ^{3}H-thymidine (1 μCi) and harvested with an automatic cell harvester (Skatron, Sterling, VA). The stimulation index (SI) was calculated by dividing the ct/min in cultures containing antigen or mitogen by the ct/min in cultures containing medium.

In some experiments, indomethacin (10 μg/ml; Sigma) or N^G-monomethyl-L-arginine (L-NMMA (0·5 mM; Calbiochem, La Jolla, CA)) or murine natural IL-2 (10 U/ml) was added to the cultures of lung cells. IL-2 was prepared as previously described from supernatants of X630mIL-2 cells [19], a kind gift of Dr T. Owens (Department of Microbiology and Immunology, McGill University), who also determined the amount of IL-2 in the supernatants by the proliferation of IL-2-dependent CTL-L cells.

DTH responses

DTH was measured as a footpad swelling after antigen exposure at 7 days post-infection. Mice were injected into the right rear footpad with 40 μl of heat-killed *Ps. aeruginosa* at 2×10^8 CFU/ml. Left footpads injected with saline served as controls. Footpad thickness was measured with calipers 24 h following injection, and the DTH response is expressed as a difference in thickness between right and left footpads.

Determination of Pseudomonas*-specific antibody*

Normal, control and *Ps. aeruginosa*-infected mice were bled via the retro-orbital plexus at 7 and 13 days post-infection. Serum from individual mice was collected and frozen until the amount of *Ps. aeruginosa*-specific antibody was determined by ELISA. The ELISA was performed using a modification of a previously described assay [20]. Briefly, flat-bottomed, 96-well plates (Immulon II; Dynatech, Chantilly, VA) were coated overnight at 4°C with 10^9 heat-killed *Ps. aeruginosa* in 0·05 M carbonate buffer pH 9·6 with 0·2% (w/v) sodium azide. The plates were washed three times with washing buffer consisting of PBS with 0·1% (v/v) Tween 20 and blocked with 5% (w/v) skim milk powder in PBS plus 0·1% v/v Tween 20 (Sigma). The plates were washed three times and aliquots of serum diluted in 1% (w/v) bovine serum albumin (BSA; Gibco-BRL) were added to the wells. After incubation at room temperature for 90 min, plates were washed three times and dilutions (as recommended by the manufacturer) of rabbit anti-mouse isotype and IgG subclass-specific horseradish peroxidase-conjugated antibodies (Southern Biotechnologies Associates Inc., Birmingham, AL) were added. The plates were incubated for 60 min at room temperature and the enzyme substrate, 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Bio-Rad, Mississauga, Ontario, Canada), was added. The plates were incubated for 20 min in the dark at room temperature and then read in an ELISA reader at a wavelength of 405 nm. For each serum sample, duplicate wells of antigen-coated and non-coated wells were prepared. Data are presented as relative OD, which was calculated by subtracting the mean OD of the non-coated wells from the mean OD of the coated wells. The mean ± s.e.m. of relative OD of groups of mice are presented.

Statistical analysis

Differences between groups of mice were analysed by Student's *t*-test. A probability of less than 0·05 was considered significant.

RESULTS

In vitro *proliferation of lymphoid cells from* Pseudomonas*-infected BALB/c and C57Bl/6 strain mice*

The *in vitro* proliferative responses of leucocytes prepared from lungs and spleens harvested from normal BALB/c and C57Bl/6 mice and from mice infected intratracheally 20 days previously with 10^4 CFU *Ps. aeruginosa* were determined. As shown in Fig. 1a, cultures of unseparated lung cells from both normal and infected BALB/c mice exhibited proliferation in response to stimulation with Con A. Moreover, the development of specific immunity to *Ps. aeruginosa* in BALB/c mice was demonstrated by the finding that unseparated lung cells from these hosts responded to heat-killed bacteria, while cells from uninfected, control BALB/c mice did not. In contrast, unseparated lung cells from normal or infected C57Bl/6 mice did not proliferate in response to either Con A or specific antigen.

Previous studies in man demonstrated that T cells are the major proliferating cells in response to *Pseudomonas* and that this response is enhanced by the presence of adherent cells [21]. However, it is also known that adherent, interstitial lung macrophages suppress T cell responses to several stimuli, and that this suppression is enhanced by infection [17,18]. In preliminary studies, we observed that non-adherent, nylon-wool enriched, lung T cells from BALB/c strain mice cultured in the presence of spleen cells from normal, syngeneic animals proliferated vigorously to *Ps. aeruginosa* antigen, while T cells depleted of adherent cells or adherent cells alone did not (data not shown). Therefore, proliferation of lung T cells from infected BALB/c and C57Bl/6 strain mice co-cultured with normal, syngeneic spleen cells, containing adherent APC, was compared (Fig. 1b). Lung T cells from normal and infected BALB/c strain mice responded to Con A, and T cells from infected but not uninfected animals responded to specific antigen. Similarly, lung T cells from both normal and infected C57Bl/6 strain mice proliferated in response to Con A, while only cells from infected mice responded, albeit a low response, to heat-killed *Ps. aeruginosa* antigen. The response of

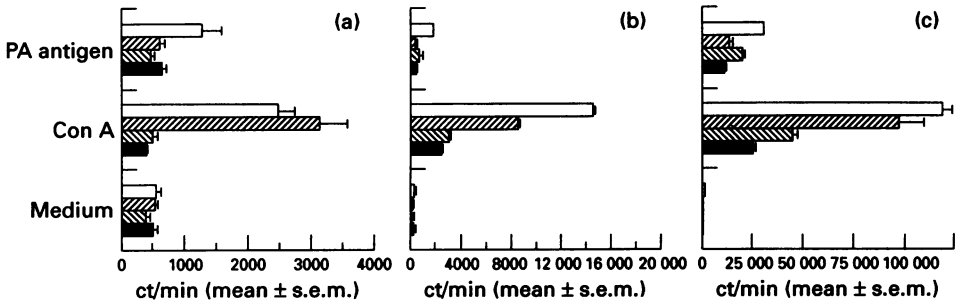

Fig. 1. Proliferative responses of lymphocytes from (a) unseparated lung cells, (b) non-adherent nylon wool-purified lung T cells cultured with syngeneic spleen cells, and (c) spleen cells from BALB/c (BALB) and C57Bl/6 (B6) mice infected intratracheally 20 days previously with approximately 10^4 colony-forming units (CFU) *Pseudomonas aeruginosa*. Lung cells from two to three infected or normal mice were pooled. The responses of spleen cells from the three individual mice were determined. Cells were stimulated *in vitro* for 48 h with concanavalin A (Con A), heat-killed *Ps. aeruginosa* or medium. Data are presented as mean ct/min ± s.e.m. of triplicate cultures for lung cells and of triplicate mice for spleen cells. The results of one of two duplicate experiments are presented. □, BALB/infected; ▨, BALB/normal; ▧, B6/infected; ■, B6/normal.

interstitial T cells from normal and infected BALB/c strain mice was consistently and significantly higher than the response of T cells from the respective group of C57Bl/6 strain mice to stimulation with Con A (normal $P < 0{\cdot}001$; infected $P < 0{\cdot}001$) and for infected mice, with antigen ($P < 0{\cdot}01$).

As shown in Fig. 1c, determination of the proliferative response of splenocytes from normal and infected C57Bl/6 and BALB/c strain mice to Con A demonstrated that cells from BALB/c strain mice had a significantly greater response than cells from C57Bl/6 strain mice ($P < 0{\cdot}001$ for both normal and infected). Unlike lung T cells, unseparated splenocytes from infected as well as normal mice of either strain responded to *Ps. aeruginosa* antigen, suggesting that this antigen has mitogenic activity, perhaps for splenic B cells. Cells from infected animals of either strain, however, exhibited a significantly greater response to specific antigen ($P < 0{\cdot}001$) than cells harvested from normal, uninfected mice, but the response to *Ps. aeruginosa* antigen of spleen cells from infected BALB/c strain mice was significantly higher ($P < 0{\cdot}001$) than the response of cells from infected C57Bl/6 mice.

Kinetic analysis of the proliferative response to specific antigen of unseparated lung and lung T cells from C57Bl/6 and BALB/c strain mice was performed at 6, 12 and 20 days post i.t. infection with 10^4 CFU *Ps. aeruginosa*. The results of this study are presented in Fig. 2 as Δct/min. As observed previously, unseparated and lung T cells from normal C57Bl/6 or BALB/c strain mice did not respond to specific antigen. Unseparated and lung T cells from BALB/c mice responded to specific antigen on days 6, 12 and 20 post-infection. The response of unseparated cells from C57Bl/6 mice was suppressed, so that there was total inhibition of the T cell response to specific antigen up to 20 days post-infection. Lung T cells from these hosts responded at all time points examined. However, the response in ct/min of lung T cells from infected BALB/c strain mice to specific antigen was 1·5–6-fold higher than cells from infected C57Bl/6 strain mice up to 20 days post-infection.

The observations of suppression of proliferation in response to the mitogen Con A and to specific antigen of unseparated lung cells from infected C57Bl/6 mice and of lower responses of lung T cells from these hosts in comparison with BALB/c strain mice following infection with 10^4 CFU *Pseudomonas* suggested that a difference in bacterial load in the lung may contribute to the differences in proliferation. To investigate this possibility, BALB/c strain mice were infected intratracheally with a 10-fold higher number of bacteria, and the proliferation of unseparated lung cells and lung T cells determined on day 6 post-infection in response to Con A and *Ps. aeruginosa* antigen.

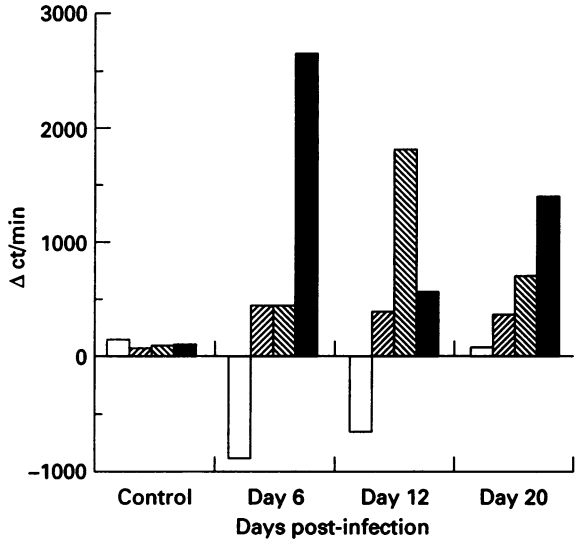

Fig. 2. Kinetics of proliferative responses to *Pseudomonas aeruginosa* antigen of unseparated lung cells and lung T cells from BALB/c (BALB) and C57Bl/6 (B6) mice infected intratracheally 6, 12 and 20 days previously with approximately 10^4 colony-forming units (CFU) *Ps. aeruginosa*. Lung cells from two to three infected or normal mice were pooled. Triplicate cultures of cells were stimulated *in vitro* for 48 h with heat-killed *Ps. aeruginosa* or medium. Data are presented as Δct/min which was calculated by subtracting the mean ct/min of cells cultured with medium only from the mean of cultures stimulated with specific antigen. Data are representative of experiments repeated four times for control mice, three times for days 6 and 12 and twice for day 20. □, B6/unseparated; ▨, B6/lung T; ▧, BALB/unseparated; ■, BALB/lung T.

Table 1. Proliferative responses of unseparated lung cells and lung T cells from BALB/c mice infected intratracheally with 10^5 CFU *Pseudomonas aeruginosa*

Lung cells harvested from	Unseparated cells			T cells		
	Medium	Con A	Antigen	Medium	Con A	Antigen
	Mean ct/min ± s.e.m.					
Normal mice	1040 ± 94	5480 ± 92 (5·3)	1036 ± 169 (1·0)	220 ± 5	13 330 ± 125* (60·6)	594 ± 60 (2·7)
Infected mice	1751 ± 153	2523 ± 154 (1·4)	133 ± 18 (0·07)	704 ± 43	30 512 ± 398* (43·3)	3116 ± 664 (4·4)

* $P < 0{\cdot}005$.

As demonstrated by the results shown in Table 1, based on a comparison of SI, unseparated lung cells from BALB/c strain mice infected with 10^5 CFU *Ps. aeruginosa* exhibited lower proliferation in response to Con A compared with cells from uninfected mice (SI 1·4 *versus* 5·3). Proliferation of these cells was totally suppressed in response to specific antigen. The response to Con A of lung T cells from infected hosts was less than normal (SI 43 *versus* 60). However, lung T cells from infected mice exhibited a significant response to specific antigen ($P < 0{\cdot}005$).

To investigate the mechanism of suppression of proliferative responses of cells from *Pseudomonas*-infected mice, the role of nitric oxide and prostaglandins, products of macrophages known to suppress lymphocyte proliferation, was investigated [22,23]. The nitric oxide inhibitor, L-NMMA (0·5 mM), indomethacin (10 μg/ml), an inhibitor of prostaglandin production, or a combination of these inhibitors was added to cultures of unseparated lung cells harvested from normal or infected BALB/c strain mice used for the experiment shown in Table 1. In agreement with an earlier observation [24], the addition of L-NMMA to cultures of unseparated lung cells from normal mice caused a small decrease, which was not significant, in proliferation compared with antigen alone (Table 2, Experiment 1). The addition of L-NMMA or indomethacin alone or in combination to cultures of cells from infected but not normal mice significantly increased the proliferative response to *Ps. aeruginosa* antigen compared with cells cultured with antigen alone. In comparison with medium, however, proliferative responses, even in the presence of the inhibitors, were not significantly increased, suggesting that abrogation by the inhibitors of antigen-stimulated suppression, caused by the infection, resulted in increased proliferative responses. The addition of IL-2 to cultures of lung cells from infected but not uninfected mice resulted in significantly increased proliferation in comparison with medium, suggesting the presence of IL-2-responsive cells in the lungs of infected BALB/c mice.

Addition of inhibitors alone or in combination, likewise, significantly increased the response to specific antigen of unseparated lung cells, but not lung T cells from C57Bl/6 strain mice infected with 10^4 CFU *Pseudomonas* in comparison with cultures containing antigen alone (Table 2, Experiment 2), suggesting that suppression is mediated by a population of adherent interstitial lung cells, probably macrophages. The addition of IL-2 to both unseparated and T cell-enriched cultures resulted in significant proliferation in comparison with medium suggesting that, during *Pseudomonas* infection, C57Bl/6 mice have IL-2-responsive T cells in the lung.

In vivo *immune responses of* Pseudomonas-*infected mice: DTH and specific antibody responses*

The *in vivo* relevance of the differences observed in proliferation *in vitro* of lung T cells from *Ps. aeruginosa*-infected BALB/c and C57Bl/6 mice was investigated by determining DTH responses on day 7, and serum levels of *Pseudomonas*-specific antibodies on days 7 and 13 after i.t. infection with 10^5 CFU *Ps. aeruginosa*. BALB/c mice had a significantly greater DTH response than that

Table 2. Suppression of proliferative responses of lung cells from *Pseudomonas aeruginosa*-infected mice by nitric oxide and PGE_2

*Experiment 1** Proliferative response to	Unseparated lung cells from Normal mice	Unseparated lung cells from Infected mice
	Mean ct/min ± s.e.m.	
Medium	1040 ± 94	1751 ± 153
Antigen	1036 ± 169	133 ± 18
Antigen + L-NMMA	680 ± 40¶	1216 ± 93‡§
Antigen + indomethacin	813 ± 29¶	859 ± 24‡§
Antigen + L-NMMA/indomethacin	660 ± 10¶	2320 ± 100‡§
IL-2	1107 ± 24§	2584 ± 209‡¶

Experiment 2† Proliferative response to	Unseparated cells	T cells
	Mean ct/min ± s.e.m.	
Medium	1478 ± 90	440 ± 49
Antigen	266 ± 3	822 ± 163¶
Antigen + L-NMMA	1284 ± 13‡	767 ± 24¶
Antigen + indomethacin	2681 ± 344‡	1190 ± 78¶
Antigen + L-NMMA/indomethacin	3727 ± 177‡	1110 ± 21¶
IL-2	3644 ± 286‡	9951 ± 336‡

* BALB/c mice infected intratracheally with 10^5 CFU *Ps. aeruginosa*.
† C57Bl/6 mice infected intratracheally with 10^4 CFU *Ps. aeruginosa*.
‡ $P < 0{\cdot}001$ compared with antigen alone.
§ Not significant compared with medium.
¶ $P < 0{\cdot}001$ compared with medium.
PGE_2, Prostaglandin E_2; L-NMMA, N^G-monomethyl-L-arginine.

Table 3. *In vivo* immune responses of *Pseudomonas aeruginosa*-infected BALB/c and C57Bl/6 mice

Group*	DTH† Mean ± s.e.m. (mm)	*Ps. aeruginosa*-specific antibodies‡ Mean relative OD ± s.e.m. IgG1	IgG2a	IgG2b	IgM	CFU‡ Mean ± s.e.m. (log_{10})
BALB/c						
Infected	0·44 ± 0·04§	0·01 ± 0·004¶	0·01 ± 0·0005	0·04 ± 0·024§	0·21 ± 0·042**	0
Normal	0·04 ± 0·06††	0	0	0·01 ± 0·0009	0·02 ± 0·005	—
C57Bl/6						
Infected	0·13 ± 0·04§	0·07 ± 0·023¶	0·02 ± 0·006	0·99 ± 0·209§	0·42 ± 0·113**	5·3 ± 0·01
Normal	0·04 ± 0·03††	0	0	0·02 ± 0·005	0	—

* Groups of two to five mice were infected intratracheally with approximately 10^5 CFU *Ps. aeruginosa*. Experiment was repeated with similar results.

† Determined on day 7 post-infection.

‡ Determined on day 13 post-infection.

§ $P < 0·001$.

¶ $P < 0·01$.

** $P < 0·05$.

†† Not significant.

of C57Bl/6 mice (Table 3). On day 7 post-infection, there were small increases in all antibody isotypes in both mouse strains, with C57Bl/6 mice having a trend towards higher levels of IgG2b and IgM than BALB/c mice (data not shown). As shown in Table 3, small increases in IgG1, IgG2a, IgG2b and IgM *Ps. aeruginosa*-specific antibodies were observed in BALB/c mice on day 13 post-infection. In C57Bl/6 mice there was likewise a small increase in IgG1 and IgG2a levels, but a marked increase in IgG2b and IgM levels. The levels of IgG1, IgG2b and IgM were significantly higher in C57Bl/6 mice compared with BALB/c mice. On day 13 post-infection, BALB/c mice, which had high DTH and low *Pseudomonas*-specific antibody responses, cleared the infection, while C57Bl/6 mice, which had low DTH and high antibody levels, had more than 10^5 CFU bacteria in the lung.

DISCUSSION

It is well established that cell-mediated immunity plays a major role in controlling infections with intracellular microorganisms, including bacteria, such as *Listeria monocytogenes*, and protozoan parasites, such as *Leishmania major*. In contrast, little attention has been focused on the role of cell-mediated immunity in host defence against bronchopulmonary infection with the Gram-negative bacterium, *Ps. aeruginosa*. Rather, most studies of protective immunity to *Ps. aeruginosa* have focused on the roles of antibody, complement and phagocytic cells, since these factors have traditionally been thought to be important in host defence against Gram-negative bacteria [4,5]. This focus is in spite of numerous studies demonstrating a positive correlation in CF patients between high titres of *Ps. aeruginosa*-specific IgG in the serum and both the aggressive course of infection and poor clinical prognosis [2,25–28]. In addition, there appears to be no evidence of a defect in non-specific inflammatory responses, involving polymorphonuclear leucocytes and macrophages, in the lungs of CF patients [5]. Rather, accumulating evidence suggests that the vigorous and chronic inflammatory response characteristic of lung disease in CF patients may contribute both to a defect in local host defences and to tissue damage in the lung.

In the present investigation, we studied *in vitro* and *in vivo* T cell responses in mice during a chronic bronchopulmonary infection with *Ps. aeruginosa* strain 508, a mucoid strain, originally isolated from the sputum of a CF patient. First, we compared the *in vitro* proliferative responses of unseparated lung cells and T cells isolated from the lung interstitium of two inbred mouse strains, BALB/c and C57Bl/6, identified as resistant and susceptible, respectively, in on-going studies in our laboratory to establish the genetic basis of resistance to chronic bronchopulmonary *Ps. aeruginosa* infection in mice. Following i.t. infection with 10^4 CFU *Ps. aeruginosa*, unseparated lung cells from infected BALB/c mice were found to have strong proliferative responses to heat-killed *Pseudomonas* or Con A up to 3 weeks post-infection, while cells from infected C57Bl/6 mice did not proliferate in response to either stimulus at any time point examined. In agreement with the results of previous studies using human peripheral blood mononuclear cells, in which T cells were identified as the major proliferating cell in response to heated-killed *Pseudomonas* [21], interstitial lung T cells from infected BALB/c or C57Bl/6 mice, cultured with syngeneic, adherent spleen cells from normal animals, were found to proliferate in response to specific antigen and Con A at various times up to 20 days post i.t. infection. It is of interest to point out, however, that the response of T cells from infected BALB/c mice was consistently observed to be significantly higher than the response of T cells from infected C57Bl/6 mice to both *Ps. aeruginosa* antigen and Con A. In addition, we observed that interstitial T cells as well as spleen cells from normal BALB/c mice exhibited a significantly higher response to Con A than cells from normal C57Bl/6 mice.

The latter observations suggest that an intrinsic difference in T cell proliferative responses between BALB/c and C57Bl/6 mice may contribute to the difference in response following *Ps. aeruginosa* infection. Alternatively, a difference in bacterial

load in the lung may contribute to suppression of T cell proliferation in susceptible C57Bl/6 mice. We examined this possibility by determining the proliferation of lung T cells from BALB/c mice following i.t. infection with a 10-fold higher dose of bacteria. We observed that unseparated lung cells from these BALB/c mice had suppressed proliferation in response to Con A and were unresponsive to specific antigen similar to C57Bl/6 mice infected with 10^4 CFU. There was a significant response to specific antigen of lung T cells from these hosts, but the response to Con A was less than normal. Addition of the nitric oxide inhibitor, L-NMMA, or the prostaglandin inhibitor, indomethacin, to cultures of unseparated lung cells from BALB/c mice infected with 10^5 CFU *Ps. aeruginosa* or C57Bl/6 mice infected with 10^4 CFU resulted in abrogation of suppression of proliferation of T cells to specific antigen. These results suggest that production of these mediators, presumably by activated lung macrophages, in part mediated the observed suppression of proliferation. Previous studies in the lung [23,29] as well as the spleen [24,30] have demonstrated a role for macrophage-derived nitric oxide and prostaglandins in suppression of lymphocyte proliferation.

Our observations thus demonstrate that interstitial lung T cells from C57Bl/6 mice, which we found to develop chronic bronchopulmonary *Ps. aeruginosa* infection, have defective *in vitro* proliferative responses to specific antigen and the mitogen, Con A. The results of the *in vitro* studies of T cell proliferation, moreover, were found to correlate with the *in vivo* immune responses determined during infection in the two mouse strains. BALB/c mice, which cleared the infection by 2 weeks, exhibited high DTH responses and low *Pseudomonas*-specific antibody responses. A similar finding of antibody hyporesponsiveness in BALB/c mice, which correlated with resistance, has been described following intracorneal infection with *Pseudomonas* [20]. In contrast, C57Bl/6 mice, which had more than 10^5 CFU *Ps. aeruginosa* in the lungs at 2 weeks post-infection, had significantly lower DTH responses and significantly higher levels of *Pseudomonas*-specific IgG1, IgG2b and IgM antibodies.

The observation in C57Bl/6 mice of a low DTH response, a measure of cell-mediated immunity, and of high levels of antibodies, particularly IgG1 and IgM, suggests a dysregulation of $CD4^+$ helper T cell responses in *Pseudomonas*-susceptible hosts. Two types of helper T cells have been defined based upon their cytokine secretion patterns and, consequently, their functional capabilities [31]. Th1 cells, which produce interferon-gamma (IFN-γ), IL-2 and tumour necrosis factor-beta (TNF-β), mediate DTH and macrophage activation, and provide B cell help for production of IgG2a antibody. Th2 cells produce IL-4, IL-5 and IL-10 and help B cells in the production of IgM, IgG1 and IgE, and thus regulate antibody-mediated responses. The functional dichotomy of Th cell function is thought to regulate the development of protective immunity *versus* disease progression in both murine models of infection and in humans, for example, in AIDS [32,33].

The relationship between the type of Th cell response and the severity of pulmonary disease in CF patients has recently been addressed, albeit to a limited extent. Moss *et al.* [34] have proposed and presented preliminary evidence that CF is a Th2 immunoinflammatory disease in which selective expansion of Th2-like cells occurs. Studies from Greally and colleagues [35] suggest that atopy is closely associated with an increased severity of pulmonary disease in CF patients. To address the relationship between the Th cell dichotomy and the development of chronic bronchopulmonary infection with *Ps. aeruginosa* in the murine model, studies are currently underway in our laboratory to determine the pattern of cytokine production by T cells (both $CD4^+$ and $CD8^+$) in the lungs of BALB/c *versus* C57Bl/6 mice. Elucidation of the relationship between the Th cell dichotomy and the severity of pulmonary disease in CF patients may result in a novel approach of immunotherapy of lung disease.

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