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A role for CD4$^+$ T cells from orally immunized rats in enhanced clearance of *Pseudomonas aeruginosa* from the lung

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SUMMARY

The role of gut-derived CD4$^+$ T cells in clearance of *Pseudomonas aeruginosa* from the lung was studied by cell transfer experiments. Mesenteric lymph node cells from unimmunized rats, or rats orally immunized with either killed *P. aeruginosa* or *Helicobacter pylori*, were transferred to naive rats which were subsequently challenged intra-tracheally with live *P. aeruginosa*. Recipients of unseparated mesenteric lymph node cells, purifed T cells or CD4$^+$ T cells, from *P. aeruginosa*-immunized donors, all exhibited enhanced bacterial clearance from the airways compared to recipients of cells from unimmunized donors. Enhanced clearance by T cells was antigen-specific as no enhanced clearance was observed by transfer of cells from donors immunized with *H. pylori*.

INTRODUCTION

Respiratory infection with *Pseudomonas aeruginosa* is a major cause of morbidity and mortality in individuals with compromised respiratory function, such as those with cystic fibrosis (CF) and chronic bronchitis. The isolation of mucoid strains of *P. aeruginosa* from the sputum of patients with CF has been associated with a poor prognosis resulting in chronic infection and death.[1] The mechanisms by which *P. aeruginosa* evades host immunity and establishes chronic infection in the lung are many,[2] and manipulation of the immune response to promote elimination of these bacteria will require a detailed understanding of the immune mechanisms involved in bacterial clearance. Antibodies may have an important role to play in elimination of *P. aeruginosa*. Indeed, there is evidence of protection by administration of monoclonal antibodies directed against flagella,[3] lipoprotein[4] or lipopolysaccharide (LPS)[5] in a mouse burn-wound model, and by anti-LPS monoclonal antibodies and opsonizing anti-alginate antibodies in guinea-pig and rodent models of *P. aeruginosa* respiratory infection.[6,7] An association between mucoid exopolysaccharide-specific opsonophagocytic killing antibody and a lack of detectable *P. aeruginosa* colonization in some older relatively healthy patients with CF indicates a protective role for this antibody.[8] However, a number of studies has demonstrated that at least some antibodies directed against *P. aeruginosa* may contribute to damage rather than protection in respiratory *P. aeruginosa* infection,[9,10] and high antibody levels correlate with poor lung function in CF patients.[11–13] Our previous studies using a rat model of respiratory *P. aeruginosa* infection demonstrated protection following oral, oral plus intra-tracheal, intra-Peyer's patch or subcutaneous immunization with killed *P. aeruginosa*.[14] At some immunizing doses enhanced bacterial clearance was elicited in the absence of detectable antibody, suggesting that non-antibody-mediated mechanisms contributed to bacterial clearance. Other studies in our laboratory have demonstrated that T cells provide protection against respiratory colonization with *Haemophilus influenzae* in a rat model.[15] These observations are consistent with those of Markham, Pier and co-workers who described protective cell-mediated immunity to intraperitoneal *P. aeruginosa* infection in mice in the absence of an antibody response.[16,17] This protection was attributed to CD8$^+$ T cells.[16,18,19] CD8$^+$ T cells from *P. aeruginosa*-immunized mice were protective on transfer to non-immune mice[20] and were demonstrated to lyse live *P. aeruginosa* by secretion of a cytokine.[18] As the role of CD4$^+$ T cells in protection against *P. aeruginosa* infection and the role of T cells in *P. aeruginosa* respiratory infection has not been addressed, we have now investigated the role of T cells and CD4$^+$ T cells in bacterial clearance in our rat model of *P. aeruginosa* respiratory infection, by transfer studies using cells from the mesenteric lymph nodes of orally immunized donor rats. The ability of transferred unseparated mesenteric lymph node cells, purified T cells and purified CD4$^+$ T cells to elicit enhanced bacterial clearance in challenged recipients was determined. We have deliberately chosen an acute infection model which mimics the initial episode rather than a chronic infection situation. An understanding of the mechanisms involved in elimination of an acute infection should allow development of effective immunization strategies which will eliminate the initial infection from the lung and therefore

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prevent the establishment of a chronic infection in susceptible individuals.

MATERIALS AND METHODS

Animals

Male, specific pathogen-free, DA rats (from the animal facility, University of Newcastle), 8–10 weeks old, were maintained under specific pathogen-free conditions until the start of the experiment and were subsequently maintained under conventional conditions. The rats were housed three to six per cage in a temperature-controlled room and were provided with commercial rat cubes and tap water *ad libitum*.

Bacteria

A mucoid *P. aeruginosa* strain (serotype 2, phagetype 21/44/109/119X/1214) was isolated from the sputum of a patient with CF. *Helicobacter pylori* (isolated from the stomach of a patient with gastric ulcer) was used for antigen-specificity studies. Bacteria were stored at −80° and recovery made from the frozen stock every 3 months. Bacteria were grown up on chocolate agar at 37°, harvested, then suspended and washed in sterile phosphate-buffered saline (PBS; Dulbecco A tablets; Oxoid Australia Pty Ltd, Hurstville, Australia). Overnight incubation was sufficient to grow the desired quantity of *P. aeruginosa*, but 72 hr incubation was necessary to obtain sufficient *H. pylori*. The bacteria were checked for purity by replating for single colony growth on nutrient agar. The concentration of bacteria was determined by optical density at 405 nm on a Titertek Multiskan photometer (Model MCC/340; Flow Labs, Australia Pty Ltd, Sydney, Australia) using standard curves for each bacteria. This gave a good approximate concentration for immediate use and was checked to greater accuracy by plating 20 μl of undiluted preparation, and serial 10-fold dilutions of the preparation, on nutrient agar and incubating overnight to determine the number of colony-forming units (CFU). This resulted in a small variation in the concentration of bacteria used (1×10^8 – 7×10^8). For immunization, bacteria were killed by incubation in 1% (w/v) paraformaldehyde for 2 hr at 37° followed by three washes with sterile PBS. The preparations were checked for total killing of bacteria by plating on nutrient agar followed by incubation at 37° for several days.

Cell transfer experimental protocol

Groups of at least six rats were set up as follows. Three groups of donor rats: (1) unimmunized; (2) orally immunized with *P. aeruginosa*; (3) orally immunized with *H. pylori*. Six groups of test rats: (1) orally immunized with *P. aeruginosa* (positive control); (2) unimmunized (negative control); (3) orally immunized with *H. pylori*; (4) recipients of cells from unimmunized donors; (5) recipients of cells from donors immunized with *P. aeruginosa*; and (6) recipients of cells from donors immunized with *H. pylori*.

Rats were immunized orally with a daily dose of 5×10^8 paraformaldehyde-killed bacteria in 0·5 ml PBS on days 1–5 and days 8–12. This dose has been established previously as optimal for oral immunization.[14] Three days later (day 15), the donor animals were killed for removal of mesenteric lymph nodes (MLN). MLN were used as these have been demonstrated to contain gut-derived activated T cells following intestinal immunization.[21] Pooled cells were prepared from each group of donor animals and, where required, purified T cells and $CD4^+$ T cells were prepared. One-sixth of each cell preparation was infused intravenously into each of six recipients in the appropriate recipient group. Using this approach recipient rats received either 6×10^7 unseparated MLN cells, $1{\cdot}5 \times 10^7$ T cells or $(1{\cdot}5\text{–}2{\cdot}0) \times 10^7$ $CD4^+$ T cells. This was considered to be the equivalent of transferring to each recipient the number of such cells from the MLN of one animal. Four days later (day 19) the rats in all six test groups were challenged with an intra-tracheal dose of 5×10^8 live *P. aeruginosa* in 50 μl of PBS. This dose of bacteria leads to pneumonia and death within 12 hr in unimmunized animals.[14] Four hours after challenge the rats were killed by barbiturate overdose and heart-bled to obtain serum which was frozen for later antibody analysis. The lungs were lavaged, *in situ*, via the trachea, five times with 2 ml of PBS. Samples of the pooled bronchoalveolar lavage (BAL) were centrifuged at 400 *g* and frozen at −20° for subsequent antibody analysis. Twenty microlitres of undiluted, and 10-fold dilutions, of BAL were plated out onto nutrient agar. After overnight incubation the number of colonies was counted and the total number of live bacteria recovered in the BAL was calculated. The results are presented as the mean ± standard error of the mean (SEM) for each group. Statistical significance of differences between groups was determined by an unpaired *t*-test (Macintosh Systat).

Preparation of MLN cells, T cells and $CD4^+$ T cells for transfer studies

MLN were removed from immunized or unimmunized animals, chopped with a scalpel blade and gently pushed through a sieve into PBS supplemented with 5% fetal calf serum (FCS) and calcium and magnesium salts (Dulbecco B ampoules; Oxoid Australia Pty Ltd). The suspension was left to stand for 10 min to allow debris to settle, and the supernatant centrifuged. The cells were washed twice, counted, and resuspended in PBS for intravenous injection (tail vein) into recipient rats.

To enrich for T cells, MLN were subjected to nylon wool separation by the method of Julius *et al.*[22] followed by panning by a modification of the method of Wysocki & Sato,[23] as previously described,[24] to remove any remaining $OX33^+$ cells. The OX33 monoclonal antibody is selective for B cells.[25] Briefly, the cells were treated with OX33 monoclonal antibody (Seralab, Immunodiagnostics, Camperdown, Australia) at a 1/50 dilution in PBS. After incubation for 30 min at 4° the cells were washed twice and panned on plastic plates (Labtek, Miles Laboratories, Sydney, Australia) coated with rabbit anti-mouse immunoglobulin (Dako Immunoglobulins, Copenhagen, Denmark).

To obtain purified $CD4^+$ T cells, nylon wool-enriched T cells were panned to remove both $CD8^+$ T cells and B cells. The cells were treated with OX33 monoclonal antibody to coat B cells, together with OX8 monoclonal antibody (Seralab) to select $CD8^+$ T cells[26] prior to panning.

Cell subset analysis

To determine the success of enrichment of cell subpopulations, aliquots of cells recovered at each stage of the separation were stained with fluorescein-conjugated antibodies for subset

analysis by flow cytometry. 1×10^6 cells were incubated for 20 min on ice with W3/25, OX8, OX19 or OX33 monoclonal antibodies (Seralab) at a 1/50 dilution in PBS. W3/25 is selective for $CD4^+$ cells and some macrophages,[27,28] and OX19 is selective for total T cells.[29] The cells were washed twice in PBS then resuspended in fluorescein-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Cappel, Westchester, PA) diluted 1/40 in PBS containing 10% heat-inactivated normal rat serum. To determine background staining by this second antibody, samples stained with the second antibody only were always included. After incubation on ice for 20 min the cells were washed twice and resuspended in 0·5 ml PBS; 0·5 ml of paraformaldehyde [2% (w/v) in PBS] was added while vortexing. The cells were run through a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) to determine the percentage of each subset present. The zero marker was set using unstained cells and the background obtained with the second antibody only was subtracted from the value obtained after staining with both first and second antibodies.

Determination of serum and BAL P. aeruginosa*-specific antibody levels by ELISA*

The assay used was an indirect ELISA which measures IgA, IgM and IgG antibodies against a polyvalent *P. aeruginosa* antigen. The antigen was prepared by suspending the bacteria harvested from 20 chocolate agar plates in 10 ml of PBS. After two washes at 1800 ***g***, bacteria were suspended in 4 ml of PBS and sonicated (Soniprep 150; MSE Scientific Instruments, Crawley, UK) using a small probe assembly (9·5-mm tip diameter). Sonication was performed with the amplitude set at 6 μ for three cycles of 30 seconds on and 60 seconds off. After sonication, the suspension was centrifuged at 10 000 ***g*** for 15 min, and the supernatant was used as the crude polyvalent antigen. The antigen was diluted 1/100 (v/v) in 0·05 M sodium carbonate–bicarbonate buffer (pH 9·6) to coat alternate rows of PolySorp plates (Nunc, Roskilde, Denmark). After overnight incubation at 4°, the plates were washed five times in PBS containing 0·05% (v/v) Tween 20 (PBS–Tween). After the addition of 100-μl portions of appropriate dilutions of the samples, standards and quality control samples, the plates were incubated for 90 min at room temperature. The dilutions were made in PBS containing 5% (w/v) milk prepared from skim milk powder (Diploma instant skim milk powder; Bonlac Foods Ltd, Melbourne, Australia). After the plates were washed five times with PBS–Tween, 100 μl of peroxidase-conjugated goat anti-rat IgG, IgM or IgA (Fc-specific; Nordic Immunological Laboratories, Tilberg, the Netherlands), diluted to an appropriate concentration in PBS containing 5% (w/v) milk, was added to each well. After incubation at room temperature for 90 min, the plates were washed five times in PBS–Tween. One hundred microlitres of substrate solution [3,3′,5,5′-tetramethyl-benzidine (Sigma Chemicals, St Louis, MO) in 0·05 M citrate–0·1 M phosphate buffer, pH 5·0, containing 0·05% (v/v) H_2O_2] was added to each well. After incubation for 30 min at room temperature the reaction was stopped by the addition of 100 μl of 0·5 M H_2SO_4. The plates were then read on a Titertek Multiskan MCC/340 (Sydney, Australia) using an automatic computer control and Titersoft program. Readings were made at 492 nm wavelength with 690 nm reference filter. The standards used were dilutions of pooled serum obtained from five rats immunized by direct Peyer's patch immunization with killed *P. aeruginosa* in incomplete Freund's adjuvant, as previously described,[14] using a total dose of 5×10^8 bacteria/rat. The highest standard concentration used was a 1/2000 dilution of this serum pool. The optical density of the highest standard concentration was arbitrarily set at 10 ELISA U/100-μl sample. A standard curve was generated by plotting the optical density against the ELISA units. Appropriate dilutions of unknown serum and BAL samples were made. For serum samples, the dilutions were 1/10–1/50 for unimmunized animals, and 1/100–1/2000 for orally immunized animals. For BAL samples the dilutions tested were 1/2 for unimmunized animals, and 1/2–1/40 for immunized animals. The antibody concentration in each unknown sample was determined from the standard curve and expressed as ELISA U/100 μl of undiluted sample.

Determination of serum and BAL H. pylori*-specific antibody levels by ELISA*

This assay was performed in an identical manner to the *P. aeruginosa*-antibody ELISA except that the plates were coated with a crude antigen prepared from sonicated *H. pylori*, and the standard serum used was obtained from rats immunized by the intra-Peyer's patch route with *H. pylori* in incomplete Freund's adjuvant.

Determination of association between enhanced bacterial clearance and survival after challenge

To validate the use of bacterial clearance as a measure of protection in this model, 18 rats orally immunized with *P. aeruginosa* and 15 unimmunized animals were challenged intratracheally with 5×10^8 live *P. aeruginosa* 14 days after completion of the oral immunization regimen. Six of each group were killed by barbiturate overdose at 4 hr, 12 hr and 36 hr to determine bacterial clearance. The remaining animals were observed until death, or 36 hr, at which time the number of bacteria in BAL was determined.

Limiting dilution analysis of frequency of antigen-specific T cells in MLN of orally immunized rats

A single-cell suspension was prepared from the pooled MLN obtained on day 15 from three rats immunized orally with killed *P. aeruginosa*, or three rats immunized orally with killed *H. pylori*, as described above. Each pool of cells was adjusted to 1×10^6/ml in RPMI tissue culture medium (Cytosystems Pty Ltd, Castle Hill, Australia) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml amphotericin (obtained as a 100 × concentrate of penicillin–streptomycin–amphotericin from ICN Biomedicals, Australasia Pty Ltd, Seven Hills, Australia), 5% FCS (CSL Ltd, Parkville, Australia), 2 mM L-glutamine, 10 mM HEPES buffer (pH 7·2) and 5×10^{-5} M 2-mercaptoethanol. Serial twofold dilutions of the cells were prepared with cells (at 1×10^6/ml) from the MLN of unimmunized rats as filler cells. Replicate cultures (24 of each dilution) were set up consisting of 1×10^5 cells/well of a flat-bottomed 96-well tissue culture plate (Nunc) with or without 0·1 μg/ml of the appropriate antigen. The antigens were prepared as described for the ELISA assays but were extensively dialysed prior to use in tissue culture. After 4 days of culture, each well was pulsed for 8 hr with 0·5 μCi [methyl-^{3}H]thymidine (specific activity 25 Ci/mmol; Amersham

International, Amersham, UK). The cultures were harvested onto a glass fibre filter, the filter disks placed in Biodegradable Counting Scintillant (Amersham, Arlington Heights, IL) and c.p.m. determined in a LKB-Wallac 1215 Rackbeta liquid scintillation counter (Wallac Oy, Turku, Finland). After subtraction of background counts (cultures without antigen) the proportion of non-responding wells was determined and the frequency of antigen-specific proliferating cells calculated.[30] This proliferation assay is specific for CD4$^+$ T cells, as removal of W3/25$^+$ (CD4$^+$) cells reduces the proliferative response by 70% but removal of OX8$^+$ (CD8$^+$) cells or OX33$^+$ cells (B cells) does not reduce the proliferative response (Dunkley *et al.*, unpublished data).

RESULTS

Enhanced bacterial clearance from BAL is associated with increased survival

The number of surviving rats at various times after intra-tracheal challenge with 5×10^8 live *P. aeruginosa* is shown in Fig. 1a. All unimmunized rats died between 11 and 12 hr after challenge, whereas all orally immunized rats were alive at this time. At 36 hr, 50% of the orally immunized rats were still alive, and these were killed at this time to determine BAL bacterial counts. An increase in the number of bacteria in the BAL of non-immune rats occurred up to the time of death, whereas a decrease in the number of bacteria in the BAL of orally immunized rats at 12 hr and 36 hr was observed (Fig. 1b). Although the differences in bacterial numbers between immune and non-immune rats were greater at 12 hr than 4 hr, 4-hr bacterial clearance assays were subsequently used as the difference between the two groups was significant at this time ($P < 0.05$) and the challenge/tissue collection procedure could be completed within 8 hr.

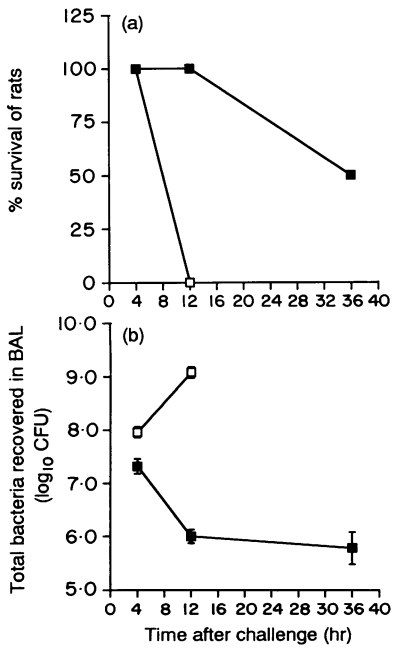

Figure 1. Recovery of bacteria from the BAL (a), and the survival (b) of unimmunized rats (□) and rats orally immunized with *P. aeruginosa* (■), at various times after intra-tracheal challenge with 5×10^8 live *P. aeruginosa*. Immunized rats were challenged 14 days after the completion of the oral immunization regimen. The values presented in (a) are the mean ± SEM of six rats. The values presented in (b) are the percentage surviving out of 12 immunized and nine non-immunized rats.

Table 1. Change in cell phenotype distribution after procedures to enrich for T cells and CD4$^+$ T cells

	% of total cell population staining with			
	W3/25	OX8	OX19	OX33
Unseparated MLN	56±3	16±2	75±3	26±3
After nylon wool	70±1	14±2	92±1	12±1
After panning (OX33$^-$) (Purified T cells)	74±1	22±1	96±1	1·4±0·4
After panning (OX33$^-$, OX8$^-$) (purified CD4$^+$ T cells)	93±2	0·9±0·5	94±1	1·0±0·6

Each value is the mean±SEM of pooled data from four experiments. Each experiment involves staining a pool of cells from six rats.

Purification of T cells and CD4$^+$ T cells

The cell phenotype distribution, after nylon wool separation to enrich for T cells and panning to enrich for CD4$^+$ T cells, is shown in Table 1. These data are the pooled data for cells from unimmunized rats and orally immunized rats, as the proportions of subsets did not differ between immune and non-immune groups. The transferred whole T-cell population was 96% OX19$^+$ (pan T cells), 74% W3/25$^+$ (CD4$^+$ T cells), 22% OX8$^+$ (CD8$^+$ T cells) and only 1·4% OX33$^+$ cells (B cells). The transferred CD4$^+$-enriched T cells were 94% OX19$^+$ and 93% W3/25$^+$, with low proportions of OX8$^+$ cells (0·9%) and B cells (1·0%).

Transfer of protection with whole MLN cells from rats orally immunized with *P. aeruginosa*

6×10^7 whole MLN cells prepared from unimmunized rats or rats orally immunized with *P. aeruginosa* were infused intravenously into each of six recipient rats per group. Four days later these rats and two control groups (rats orally immunized with *P. aeruginosa* 19 days previously, and unimmunized rats) were challenged intra-tracheally with 5×10^8 live *P. aeruginosa*. The bacterial clearance in the BAL of the recipients of unseparated MLN cells and in the BAL of control immunized and unimmunized animals is shown in Fig. 2a. Compared to unimmunized rats, significant clearance of bacteria from BAL was observed in the immunized rats and the recipients of immunized MLN cells. In BAL, only *P. aeruginosa*-specific IgG antibody was significantly elevated in immunized compared to unimmunized rats (Fig. 2b). In serum, significantly elevated *P. aeruginosa*-specific IgG, IgA and IgM was only detected in immunized rats and in recipients of MLN

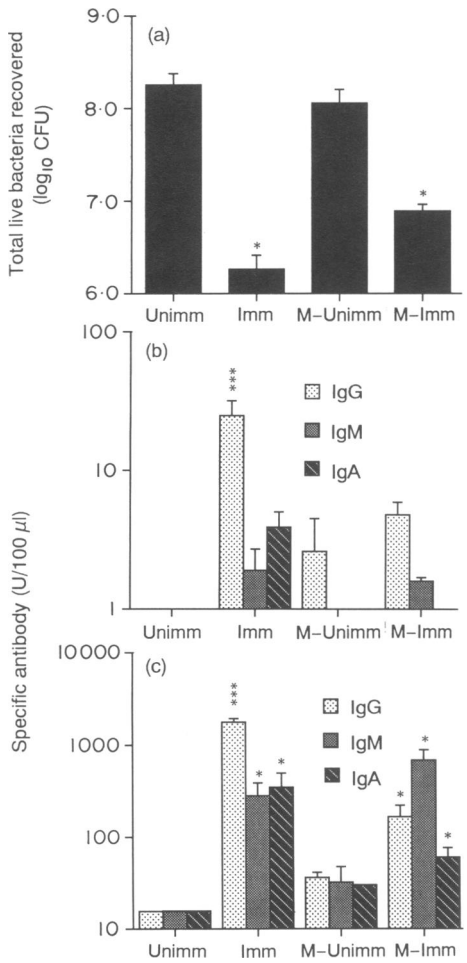

Figure 2. The number of live *P. aeruginosa* recovered from BAL (a), and the *P. aeruginosa*-specific antibody levels in BAL (b) and serum (c) 4 hr after intra-tracheal challenge with 5×10^8 live *P. aeruginosa* in unimmunized rats (Unimm), rats orally immunized with *P. aeruginosa* (Imm), and recipients of unseparated MLN cells from either unimmunized rats (M-Unimm) or rats orally immunized with *P. aeruginosa* (M-Imm), as described in the cell-transfer protocol. The values presented are the mean ± SEM (six rats/group). The significance of the difference compared to the unimmunized controls is $*P < 0{\cdot}05$, $***P < 0{\cdot}005$.

from immune rats (Fig. 2c). The antibody in the recipients was presumably due to synthesis by B cells among the transferred MLN. Further experiments were then performed to see if protection could be transferred with purified T cells, where any effect of antibody produced by transferred B cells would be eliminated.

Transfer of protection with purified T cells from rats orally immunized with *P. aeruginosa*

$1{\cdot}5 \times 10^7$ purified T cells, prepared from unimmunized rats or rats orally immunized with *P. aeruginosa*, were injected intravenously into each of six recipient rats per group. Four days later these rats and two control groups (rats orally immunized with *P. aeruginosa* 19 days previously, and unimmunized rats) were challenged by intra-tracheal instillation of 7×10^8 live *P. aeruginosa*. Four hours later the clearance of *P. aeruginosa* from BAL was measured (Fig. 3a). When compared with unimmunized control rats, significant clearance of *P. aeruginosa* from BAL was observed in the rats orally immunized with *P. aeruginosa* and the recipients of T cells from rats orally immunized with *P. aeruginosa*. The difference between the recipients of T cells from immunized animals and the recipients of cells from unimmunized animals was also significant ($P < 0{\cdot}05$). *Pseudomonas aeruginosa*-specific antibody levels (Fig. 3b,c) in both BAL and serum were only significantly elevated in the immunized rats. Specific antibody levels in recipients of transferred cells were low to undetectable and not significantly different to the unimmunized controls.

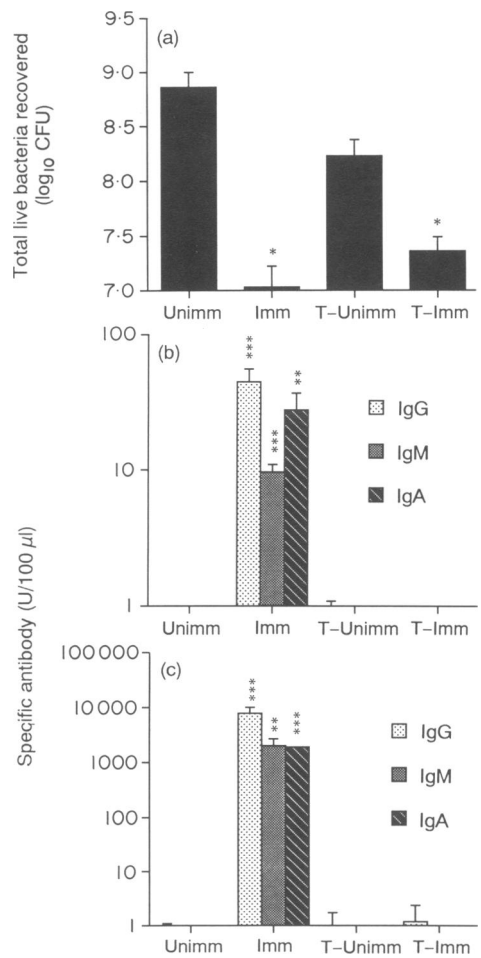

Figure 3. The number of live *P. aeruginosa* recovered from BAL (a), and the *P. aeruginosa*-specific antibody levels in BAL (b) and serum (c) 4 hr after intra-tracheal challenge with 7×10^8 live *P. aeruginosa* in unimmunized rats (Unimm), rats orally immunized with *P. aeruginosa* (Imm), and in recipients of purified MLN T cells from either unimmunized rats (T-Unimm) or rats orally immunized with *P. aeruginosa* (T-Imm), as described in the cell-transfer protocol. The values presented are the mean ± SEM (six rats/group). The significance of the difference compared to the unimmunized controls is $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}005$.

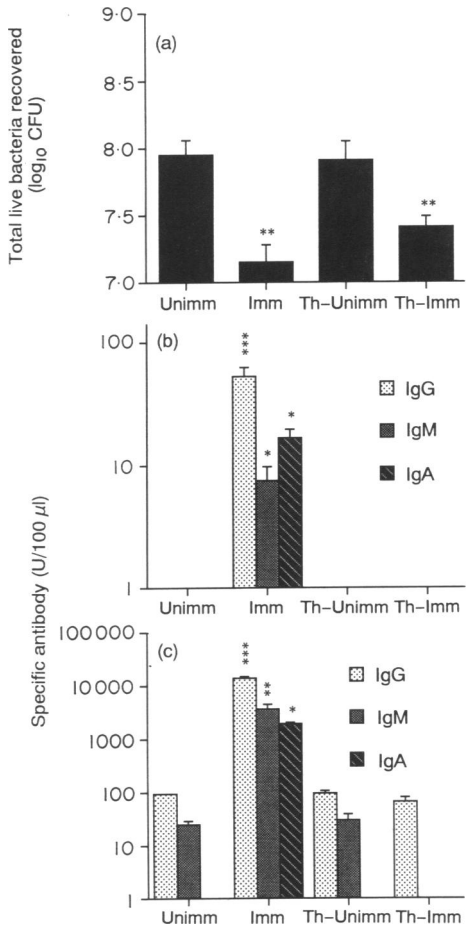

Figure 4. The number of live *P. aeruginosa* recovered from BAL (a), and the *P. aeruginosa*-specific antibody levels in BAL (b) and serum (c) 4 hr after intra-tracheal challenge with 1×10^8 live *P. aeruginosa* in unimmunized rats (Unimm), rats orally immunized with *P. aeruginosa* (Imm), and in recipients of purified MLN CD4$^+$ T cells from either unimmunized rats (Th-Unimm) or rats orally immunized with *P. aeruginosa* (Th-Imm), as described in the cell-transfer protocol. The values presented are the mean ± SEM (12 rats/group). The significance of the difference compared to the unimmunized controls is $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}005$.

Transfer of protection with purified CD4$^+$ T cells from rats orally immunized with *P. aeruginosa*

Purified CD4$^+$ T cells were prepared from unimmunized rats or rats orally immunized with *P. aeruginosa* (pooled cells from six rats/group in exp. 1 and nine rats/group in exp. 2). In each experiment the cells obtained were injected into six recipients per group ($1{\cdot}45 \times 10^7$ cells/rat in exp. 1 and $2{\cdot}0 \times 10^7$ cells/rat in exp. 2). Four days later these rats and two control groups (rats orally immunized with *P. aeruginosa* 19 days previously, and unimmunized rats) were challenged by intra-tracheal instillation of 1×10^8 live *P. aeruginosa*. The results presented in Fig. 4 are the pooled data from the two experiments (12 rats/group). Compared to the unimmunized controls, significant clearance of *P. aeruginosa* from BAL of immunized rats and recipients of CD4$^+$ T cells from immunized rats was observed (Fig. 4a). The *P. aeruginosa*-specific antibody levels in BAL and serum are shown in Fig. 4b,c. Significantly elevated specific antibody was only found in the immunized rats. Antibody levels in serum and BAL of recipients of CD4$^+$ T cells were not significantly different to those of unimmunized rats.

Non-cross-reactivity between *P. aeruginosa* and *H. pylori*

Previous studies have shown some cross-reactivity with respect to protection between *P. aeruginosa*, non-typable *Haemophilus influenzae* and *Klebsiella pneumoniae* in that immunization with *P. aeruginosa* causes enhanced clearance of the latter two bacteria from the lung.[14] *Haemophilus influenzae* and *K. pneumoniae* were therefore considered to be unsuitable controls for specificity studies. *Helicobacter pylori* was tested for cross-reactivity with *P. aeruginosa*. Figure 5a shows that, in contrast to rats orally immunized with *P. aeruginosa*, rats orally immunized with *H. pylori* did not clear live *P. aeruginosa*

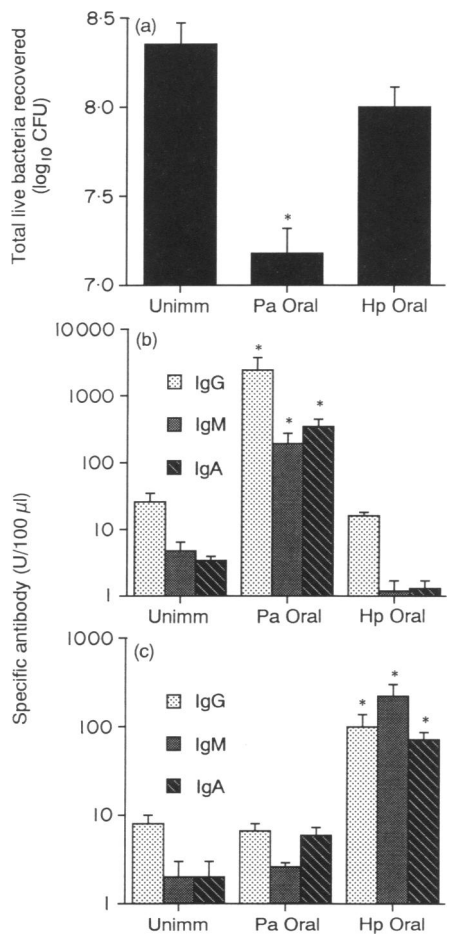

Figure 5. The number of live *P. aeruginosa* recovered from BAL (a), and the *P. aeruginosa*-specific antibody (b) and *H. pylori*-specific antibody (c) in serum 4 hr after intra-tracheal challenge with 5×10^8 live *P. aeruginosa* in unimmunized rats (Unimm) or rats orally immunized with either *P. aeruginosa* (Pa Oral) or *H. pylori* (Hp Oral). Challenge was performed 14 days after completion of the oral immunization regimen. The values presented are the mean ± SEM (six rats/group). The significance of the difference compared to the unimmunized controls is $*P < 0{\cdot}05$.

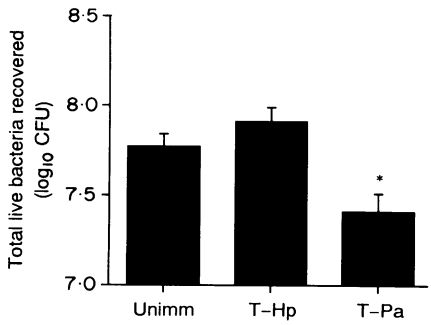

Figure 6. The number of live *P. aeruginosa* recovered 4 hr after intra-tracheal challenge with 5×10^8 live *P. aeruginosa*, from the BAL of unimmunized rats (Unimm), recipients of T cells from rats orally immunized with *H. pylori* (T-Hp) or recipients of T cells from rats orally immunized with *P. aeruginosa* (T-Pa), as described in the cell-transfer protocol. The values presented are the mean ± SEM (seven rats/group). The significance of the difference compared to the unimmunized controls is $*P < 0{\cdot}05$.

from the lung. Furthermore, there was no cross-reactivity between the antibodies produced (Fig. 5b,c) or the *in vitro* proliferative response to antigen following intestinal immunization with *H. pylori* or *P. aeruginosa* (M. Dunkley, unpublished data). *Helicobacter pylori* was therefore chosen for antigen-specificity studies.

Transfer of T cells from *H. pylori*-immunized rats does not confer protection

Figure 6 shows that, in contrast to T cells from rats orally immunized with *P. aeruginosa*, T cells from rats immunized with *H. pylori* did not elicit enhanced clearance of live *P. aeruginosa* from the lung of challenged non-immune recipients. The lack of protection by cells from *H. pylori*-immunized rats was not due to a lower number of antigen-specific cells, as the frequency of *H. pylori*-specific T cells in the MLN of *H. pylori*-immunized rats determined by limiting dilution analysis was 4×10^{-5} and the frequency of *P. aeruginosa*-specific T cells in the MLN of *P. aeruginosa*-immunized rats was $3{\cdot}7 \times 10^{-5}$. No *P. aeruginosa*-specific or *H. pylori*-specific antibody was detected in the serum or BAL of the recipient rats (data not shown).

DISCUSSION

Following intra-tracheal challenge with $1{-}7 \times 10^8$ live *P. aeruginosa*, the number of bacteria in the airways of unimmunized rats increased rapidly leading to death within 12 hr. Rats orally immunized with *P. aeruginosa* were able to clear a considerable proportion of the challenge dose of bacteria, resulting in enhanced survival. The protection was not as good as that observed previously after intra-Peyer's patch immunization, where all immunized rats survived at least a week after live bacterial challenge.[14] It is apparent, however, that the ability of orally immunized rats to clear bacteria from the airways is associated with enhanced survival, and we have therefore used bacterial clearance as a measure of the effectiveness of immunization or cell transfer.

The present study demonstrates a significant contribution to enhanced bacterial clearance from the airways by T cells and $CD4^+$ T cells from rats orally immunized with *P. aeruginosa* in circumstances where no significant elevation of *P. aeruginosa*-specific antibody can be detected. This implies that T-cell-mediated antibody-independent mechanisms of clearance occur. The manner in which transferred $CD4^+$ cells cause clearance is yet to be defined, but is via an antigen-specific mechanism as transferred cells from rats immunized with non-cross-reacting bacteria (*H. pylori*) were not effective in inducing enhanced clearance. We propose that one mechanism of action of T cells may be via activation of neutrophils, as we have previously shown that the number and phagocytic activity of these in the bronchial washings of *P. aeruginosa*-immunized animals is increased compared to that in challenged sham immunized animals following intra-tracheal challenge with live bacteria.[31] Following intestinal immunization, *P. aeruginosa*-specific $CD4^+$ cells presumably migrate from the gut-associated lymphoid tissue to the lung, where they reside until they are reactivated upon intra-tracheal challenge with live *P. aeruginosa*. Upon reactivation secreted cytokines may initiate, or contribute to, a sequence of events leading ultimately to increased neutrophil recruitment and enhanced bacterial clearance. There is evidence from *in vitro* studies that neutrophils may be activated by exposure to T cells (reviewed in ref. 32), and the T-cell cytokines lymphotoxin (tumour necrosis factor-β) and interferon-γ have been demonstrated to stimulate enhanced neutrophil-mediated killing of *Plasmodium falciparens*.[33] Another mode of action of $CD4^+$ T cells may be via a direct effect on the bacteria, as has been demonstrated for $CD8^+$ T cells.[18]

Determination of the precise role of antibody, T cells, and other cell types in protection against *P. aeruginosa* infection will aid the development of successful vaccines. Development of optimal T-cell responses by appropriate immunization strategies may be particularly important given their role in antibody production, neutrophil activation, and their direct effects on bacteria. A report of specific diminished T-cell responses in CF patients in the latter stages of infection[34] demonstrated that specific suppression of the T-cell response occurs in this disease. Boosting of the specific T-cell immunity in CF patients prior to infection may prevent the synergistic effect of increasing bacterial load and decreasing T-cell function, which lead to more severe infection and death.

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