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Effector Mechanisms of Intestinally Induced Immunity to *Pseudomonas aeruginosa* in the Rat Lung: Role of Neutrophils and Leukotriene B_4

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This paper investigates the effector mechanisms of immune clearance in the lungs of rats immunized against mucoid *Pseudomonas aeruginosa*. After the gut-associated lymphoid tissue was primed and after a subsequent pulmonary challenge with live bacteria, significantly accelerated bacterial clearances from the lung and raised levels of anti-*P. aeruginosa* antibodies in sera (immunoglobulin G [IgG], IgA, and IgM) and bronchoalveolar lavages (IgG and IgA) were observed for all immune animals. These changes were associated with enhanced recruitment, chemotaxis, chemokinesis, phagocytic indices, and chemiluminescence of pulmonary polymorphonuclear neutrophils (PMN). In the alveolar spaces of immune animals, an increase in the level of PMN recruitment was not associated with higher levels of leukotriene B_4 (LTB_4). In contrast, in nonimmune animals that were intratracheally infected with *P. aeruginosa*, the levels of recruitment and activity of alveolar PMN were lower than those in immune rats but PMN infiltration correlated with a significant increase in the synthesis of LTB_4 in the alveolar space. In pulmonary tissue, LTB_4 synthesis for both groups was elevated. These findings suggest that accelerated clearance of mucoid *P. aeruginosa* from the lungs of intestinally immunized rats is due at least in part to factors that induce the enhancement of PMN recruitment and activity in the alveolar space. The mediators that regulate this enhanced response remain unknown but do not seem to include LTB_4. The high levels of LTB_4 measured in the bronchoalveolar lavages and pulmonary tissues from nonimmune animals infected with live bacteria implicate LTB_4 as an important amplifier of the inflammatory response during acute pulmonary infections with mucoid *P. aeruginosa* in unimmunized hosts.

Pseudomonas aeruginosa is an extracellular opportunistic pathogen which causes fatal infections in compromised individuals such as burn victims and patients with cystic fibrosis (CF) (15, 36). In CF patients, colonization with *P. aeruginosa* transits from a nonmucoid to a mucoid form and eventually leads to severe pulmonary disease and death (26). Because of the poor prognosis of pulmonary colonization with *P. aeruginosa* in CF patients, a number of investigators have undertaken to assess the prophylactic value of a *P. aeruginosa* vaccine in an attempt to prevent the initial establishment of the pathogen (2, 13, 23, 32, 34, 35, 38). However, protection with a vaccine against pulmonary colonization by *P. aeruginosa* in humans has not yet been achieved. Our laboratory has developed a rat model in which intestinal immunization with killed mucoid *P. aeruginosa* cells promotes an increase in bacterial clearance from the lung and enhanced survival following a subsequent pulmonary challenge with live bacteria (2); however, the mechanisms providing anti-*P. aeruginosa* protection remained undefined. Colonization with *P. aeruginosa* induces a strong humoral immune response both in serum and in salivary and pulmonary secretions. These antibodies are elicited against a variety of *P. aeruginosa* antigens (5, 6, 19, 21, 22, 33, 43), but they are unable to stop the spread of the bacterium in the lung (4, 8, 10). In keeping with these findings, production of *P. aeruginosa*-specific antibodies does not correlate with accelerated bacterial clearance from the lungs in intestinally immunized rats (2). Clearly, other effector mechanisms of immunity are involved in the host defense against *P. aeruginosa*, and cell-mediated immunity may play a major role in this protection (24, 34, 44, 46). In an attempt to further define the effector mechanisms of pulmonary immunity to *P. aeruginosa*, the aim of the present study was to determine whether the acceleration in bacterial clearance from the lung induced by intestinal immunization was associated with enhancement of recruitment, function, and activity of polymorphonuclear neutrophils (PMN). The results indicate that (i) priming of the gut-associated lymphoid tissue with mucoid *P. aeruginosa* results in enhanced recruitment, chemotaxis, chemokinesis, and phagocytic activity of PMN following a challenge infection, (ii) these changes correlate with enhanced bacterial clearance from the lung, and (iii) leukotriene B_4 (LTB_4) synthesis does not appear to be associated with the increased PMN recruitment in the alveolar spaces of immunized rats.

MATERIALS AND METHODS

Animal model. Male, specific-pathogen-free DA rats, 8 to 10 weeks old and weighing 175 to 250 g (mean [$\bar{x}$] ± standard error of the mean [SEM] = 225 ± 5 g), were used in all experiments as described previously (2). All animals were maintained in a specific-pathogen-free environment until the start of the experimental protocol. The rats were fed commercial rodent pellets and given water ad libitum. Mainte-

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TABLE 1. Bacterial recoveries and PMN counts in bronchoalveolar lavages from nonimmune and immune rats[a]

Time of pulmonary challenge (h) and rat group	Log_{10} CFU in lungs (% clearance)	Bronchoalveolar PMN	
		%	Total no. (*P*)
0.5			
Nonimmune (n = 6)	7.58 ± 0.05	3.1 ± 0.4	$1.3 \times 10^3 \pm 0.3 \times 10^3$
Immune (n = 6)	7.11 ± 0.06 (66%)[b]	10.1 ± 1.2[b]	$1.9 \times 10^3 \pm 0.3 \times 10^3$ (0.2)
4			
Nonimmune (n = 22)	7.81 ± 0.06	65.1 ± 2.2	$3.0 \times 10^5 \pm 1.0 \times 10^5$
Immune (n = 15)	6.13 ± 0.16 (97%)[b]	93.3 ± 1.2[b]	$8.0 \times 10^6 \pm 2.1 \times 10^{6}$[b]

[a] Pulmonary challenges were with live *P. aeruginosa* cells. Lavages from nonimmunized control animals (n = 6) challenged (for 4 h) with PBS and from immunized, PBS-challenged (for 4 h) animals (n = 6) contained 1.9% ± 1.0% and 2.3% ± 1.1% PMN, respectively. Values are $\bar{x}$s ± SEMs.
[b] $P < 0.001$ compared with value for nonimmune animals.

nance of animals and experimental procedures were carried out in accordance with the guidelines of the Ethics Committee of the University of Newcastle (Newcastle, New South Wales, Australia).

Bacteria. A mucoid *P. aeruginosa* strain, serotype II, phage type 21/44/109/119×/1214 (*Pseudomonas* Reference Laboratories, London, United Kingdom), which was isolated from a patient with CF, was used in all experiments. Bacteria were grown on chocolate agar plates overnight at 37°C, harvested, suspended and washed in sterile phosphate-buffered saline (PBS; pH 7.3, 0.15 M NaCl), quantitated, and checked for purity by being replated for single-colony growth on chocolate agar. For immunization, bacteria were killed by exposure to 1% paraformaldehyde for 2 h and washed three times in sterile PBS.

Immunization protocol. It has been previously established that intra-Peyer's patch (IPP) immunization induces immune mechanisms which are independent of those seen after intraperitoneal immunization (7). For the purpose of the present study, paraformaldehyde-killed *P. aeruginosa* cells were suspended in sterile PBS to a concentration of 10^{10} CFU/ml as measured by the optical density at 405 nm determined by a Titertek Multiskan photometer (model MCC/340; Flow Laboratories, Australasia Pty., Sydney, Australia). This solution was emulsified with an equal volume of incomplete Freund's adjuvant, and 1 to 2 μl of the preparation was injected subserosally with a 27-gauge needle into each Peyer's patch of rats (immune rats) anesthetized with 3.6% chloral hydrate intravenously as described previously (18). A second group of sham-treated animals (nonimmune rats) was inoculated with incomplete Freund's adjuvant-PBS only. A third group of animals (control rats), which was used in parts of this study, was not immunized.

Bacterial clearance. Fourteen days after immunization, rats were challenged intratracheally via an intravenous catheter (20 gauge by 2 in. [ca. 5 cm]) with 50 μl of PBS containing 5×10^8 CFU of live autologous *P. aeruginosa* cells (harvested from an overnight culture on chocolate agar). Nonimmunized control animals were given intrapulmonary PBS only. At 30 min or 4 h after intratracheal inoculation, all rats were killed with an intraperitoneal overdose of Pentabarbitone sodium (Nembutal; Boehringer Ingelheim, Artarmon, New South Wales, Australia). After challenge, blood samples were obtained by cardiac puncture and bronchial washings were collected by five sequential lavages with 2 ml of sterile PBS. Each animal's descending aorta was severed, and whole lungs were perfused with heparinized (20 U of heparin sodium per ml, porcine mucous; Fisons, Thornleigh, New South Wales, Australia), sterile PBS via the right atrium to remove blood contents, excised, weighed, and frozen at −70°C until further needed. Serum aliquots were frozen and stored at −70°C until further study. Viable *P. aeruginosa* cells recovered in the bronchoalveolar lavages were counted by serial dilution and culture on nutrient agar for 18 h at 37°C. Bacterial clearance was expressed as log_{10} CFU and, for the immune group, as the percentage of cleared bacteria compared with the number of organisms recovered from nonimmune animals (Table 1). As established previously in experimental infections with *P. aeruginosa* (2) and in other systems (47), bacterial recovery from bronchoalveolar lavages is a reliable parameter which correlates well with total pulmonary bacterial clearance.

Anti-*P. aeruginosa* antibodies. Anti-*P. aeruginosa* antibodies (immunoglobulin A [IgA], IgG, and IgM) were assayed after challenge from serum and bronchoalveolar lavage samples by an enzyme-linked immunosorbent assay (ELISA) as described previously (47), with some modifications. Briefly, wells were coated with a *P. aeruginosa* sonicate diluted 1:2,000 in sodium carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Serum and bronchoalveolar lavage samples were diluted in diluent buffer (5% skim milk in 0.05% Tween 20-PBS) at the following concentrations: sera from control and nonimmune rats, 1:100 (IgG) and 1:10 (IgA and IgM); sera from immune rats, 1:2,000 (IgG) and 1:1,000 (IgA and IgM); bronchoalveolar lavages from control and nonimmune rats, 1:2 (IgG, IgA, and IgM); bronchoalveolar lavages from immune rats, 1:100 (IgG), 1:40 (IgA), and 1:20 (IgM). All samples were assayed simultaneously for the various antibody levels. Peroxidase-conjugated goat anti-rat IgA, IgG, and IgM (Nordic) were bound to the antibody-antigen complexes in individual wells, and 3,3′,5,5′-tetramethylbenzidine substrate (Sigma, Bioscientific Ltd., Sydney, Australia) was added. A_{492} was read on a Titertek Multiskan photometer against a standard curve obtained from hyperimmune rat serum. The results were expressed in ELISA units per milliliter of sample, as described previously (47).

PMN counts and phagocytic indices. Fresh 100-μl samples of each bronchoalveolar lavage were spun for 10 min at 20 × *g* onto a microscope slide with a cytospin (Shandon Inc., Pittsburgh, Pa.), and cytocentrifuged specimens were fixed and stained in Diff Quick (Vet. Med. Surg. Suppl. Pty. Ltd., Maryville, Australia). The percentages of alveolar PMN were calculated from three differential counts on each slide. The slides were washed to remove free extracellular bacteria not associated with phagocytes, and the preparations were further evaluated under light microscopy for the determination of two phagocytic indices, i.e., (i) the percentage of

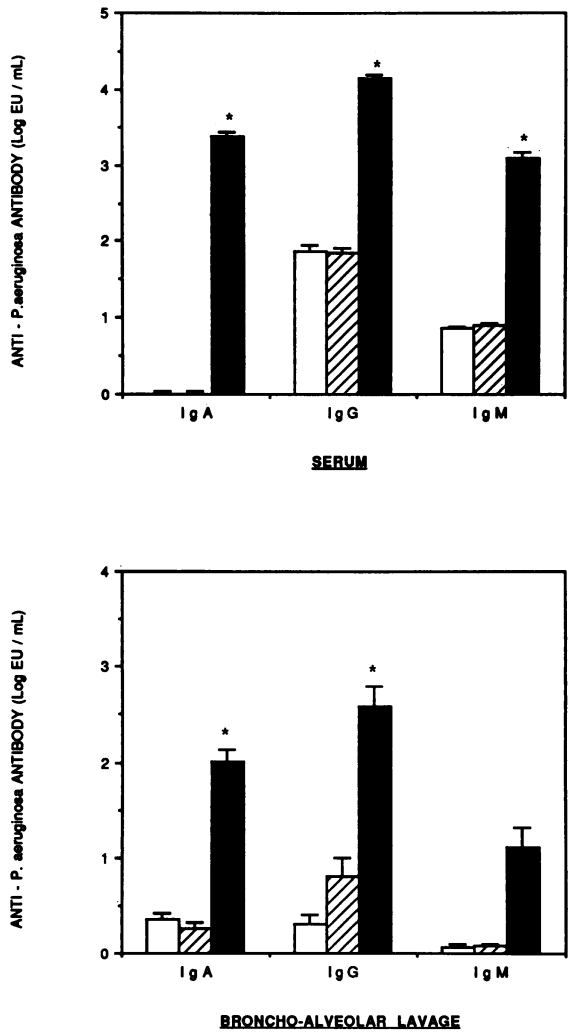

FIG. 1. Anti-*P. aeruginosa*-specific IgA, IgG, and IgM antibodies in sera and bronchoalveolar lavages of control (□), nonimmune (▨), and immune (■) rats 14 days after IPP inoculation. Values are $\bar{x}$s ± SEMs for six animals in each group. *, $P < 0.001$ compared with values for nonimmune and control rats. EU, ELISA units.

PMN that phagocytosed 1 to 5 bacteria and (ii) the percentage of PMN that phagocytosed >5 bacteria.

PMN purification. The bronchoalveolar lavages were centrifuged for 10 min at 250 × *g* and 5°C. The supernatant was frozen and stored at −70°C until further study. The pellet was emulsified and washed in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered, Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS [pH 7.2]; GIBCO Laboratories, Grand Island, N.Y.) and resuspended in 2 ml of HBSS. Erythrocytes were lysed in hypotonic HBSS for 30 s, and the remaining leukocytes in HBSS were layered over a gradient of 55, 65, and 81% Percoll (Pharmacia, Uppsala, Sweden) and centrifuged for 30 min at 2,000 × *g* and 10°C. PMN were harvested and resuspended in 1 ml of HBSS. Cells were evaluated for viability by trypan blue (0.1%) exclusion, counted with a hemocytometer, and adjusted to a final concentration of 1.25×10^6 cells per ml, and the extract was assessed for purity microscopically with a cytospin preparation as described above.

Electron microscopy. Phagocytosis of bacteria by PMN was verified under transmission electron microscopy. Purified PMN were centrifuged for 10 min at 250 × *g* and 5°C, fixed overnight in Karnovski's fixative at 5°C, postfixed for 2 h in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Spurr's medium (FSE, Sydney, Australia). Sections (80 nm each) were stained with saturated uranyl acetate in 50% ethanol and 0.04% lead citrate. Micrographs were obtained with a Philips CM 10 transmission electron microscope at an acceleration voltage of 80 kV.

Chemiluminescence. The level of phagocytic activation in alveolar PMN was assessed from 1.9×10^5 cells in 150 μl of HBSS by luminol-enhanced chemiluminescence as described previously (1, 47). Because of the lower numbers of alveolar PMN recovered from nonimmune animals (Table 1), PMN from four to nine nonimmune rats had to be pooled for the chemiluminescence and functional assays. Cells from immune animals or from pools of nonimmune animals were maintained in a light-proof Wallac luminometer (model 1251; LKB, Stockholm, Sweden), with continuous mixing, at 37°C, in prewarmed luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma, Bactolabs, Sydney, Australia), which was diluted 1:250 in HBSS from a 0.002 M solution in dimethyl sulfoxide. Zymosan (Sigma), diluted 1:8 in HBSS from an 80-mg/ml HBSS stock solution, was opsonized with pooled sera from immune or nonimmune rats and incubated for 30 min in a shaking water bath at 37°C. Immediately after the addition of opsonized zymosan to the cell-luminol preparation, chemiluminescence was read from 2-s integrations at 2, 5, 7, 12, and 17 min.

PMN function. PMN random migration, chemotaxis, and chemokinesis were measured with Boyden chambers (Medos, Sydney, Australia) and 3.0-μm-pore-size membrane filters (type SS; Millipore Corp., Bedford, Mass.). Samples (100 μl each) from the cell preparation were placed on the filters in the upper wells of each of five chambers set up in one of the following manners: (i) bottom and top of chamber filled with HBSS for calculation of random migration; (ii) bottom of chamber filled with casein (2.5 mg of HBSS per ml) and top of chamber filled with HBSS, for nonspecific chemotaxis; (iii) bottom of chamber filled with 10^8 live homologous *P. aeruginosa* cells per ml in HBSS and top of chamber filled with HBSS, for specific chemotaxis; (iv) bottom and top of chamber filled with 5% immune serum in HBSS for chemokinesis; and (v) as above, but with 5% serum from nonimmune rats. After incubation for 60 min at 37°C in an atmosphere containing 5% CO_2, the membrane filters were removed, fixed in absolute alcohol, stained with Harris hematoxylin, washed in distilled water, dehydrated in ethanol, stored overnight in xylene, and mounted between slide and coverslip. The leading fronts of migrating PMN were measured under light microscopy as described previously (52). The depths of penetration of the three leading PMN from 10 different sites were determined with a micrometer incorporated in the fine focus of a Zeiss light microscope, and the means were calculated. To avoid observer bias, the filters were coded prior to examination.

LTB_4 assay. Perfused lung specimens were thawed, homogenized in 1 ml of phosphate buffer (pH 7.4, 4°C) per g of tissue, and centrifuged for 2 min at 14,000 rpm on a benchtop microcentrifuge (Microfuge 12; Beckman Instruments Inc.). LTB_4 activity was assessed from nondiluted and diluted (1:100) samples from each serum and bronchoalveolar lavage specimen and from 1:100 and 1:250 dilutions of lung homogenate supernatants by using a competitive enzyme immunometric assay kit according to the instructions of the manufacturer (Cayman Chemical Company, Sapphire Bioscience,

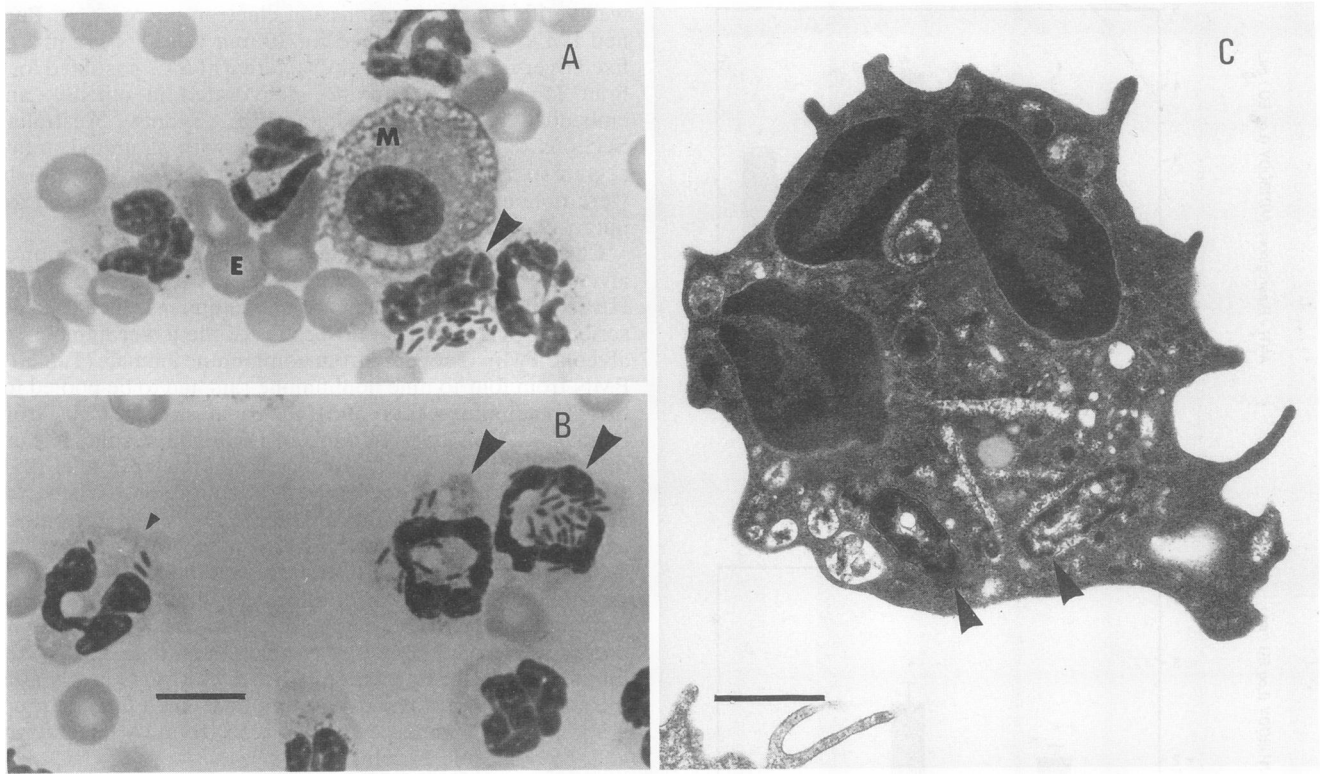

FIG. 2. (A and B) Light micrographs of cytospin preparations from the bronchoalveolar lavages of immune rats challenged for 4 h with live *P. aeruginosa* cells. PMN that have phagocytosed 1 to 5 bacteria (small arrowheads) or >5 bacteria (large arrowheads), alveolar macrophages (M), and erythrocytes (E) are easily identifiable. Bar, 10 μm. (C) Transmission electron micrograph of a PMN obtained from the bronchoalveolar lavage of an immune rat challenged for 4 h with live *P. aeruginosa* cells. Bacteria (arrowheads) in various stages of decomposition are seen within the cytoplasm of the phagocyte. Bar, 1 μm.

Alexandria, New South Wales, Australia). Briefly, the assay is based on the competition between free LTB_4 and acetylcholinesterase-linked LTB_4 tracer for limited specific rabbit antiserum-binding sites. Rabbit antiserum LTB_4 complexes, either free or tracer, are bound to a mouse monoclonal anti-rabbit antibody previously adsorbed onto the well. After 50 min of development in acetylcholinesterase-sensitive Ellman's reagent (Cayman Chemical Company), plates were read at 414 nm on a Titertek Multiskan photometer. The amount of free LTB_4 in each well is inversely proportional to the amount of LTB_4-bound tracer detected by the reagent. The assay specificity is 100% for LTB_4, 0.03% for 5(*S*)-HETE, and <0.01% for leukotrienes C_4, D_4, E_4, and F_4.

Statistical analysis. The results were expressed as $\bar{x}$s ± SEMs and compared by one-way analysis of variance, followed by Tuckey's test for multiple-comparison analysis when applicable. When results were expressed as percentages, all values underwent an arcsine transformation prior to statistical comparison and were transformed back to percentages for the expression of the $\bar{x}$s ± SEMs. Levels at which P was < 0.05 were considered significant.

RESULTS

Bacterial clearance and PMN counts. As illustrated in Table 1, IPP immunization with killed bacteria resulted in significantly enhanced bacterial clearance from the lung following a subsequent pulmonary challenge with live *P. aeruginosa*. After 0.5 h of challenge, immunized animals showed a reduction in the numbers of bacteria recovered from their bronchoalveolar lavages of 66%, compared with nonimmune rats. After 4 h, the immunized rats had cleared 97% of the bacteria. After 4 h of challenge, the enhanced clearance rate in immune animals was associated with an increase of more than 25-fold in the number of bronchoalveolar PMN over that in nonimmune challenged rats. PMN represent a small portion of the bronchoalveolar cell population in normal rat lungs (1.9% ± 1.0%) and in the lungs of immune but unchallenged animals (2.3% ± 1.1%). Live bacterial challenge in nonimmune rats induced PMN to become the bulk of the alveolar cell population (0.5 h, 3.1% ± 0.4%; 4 h, 65.1% ± 2.2%), and the proportion of PMN in rats that had been previously immunized was significantly higher (0.5 h, 10.1% ± 1.2%; 4 h, 93.3% ± 1.2%) (Table 1).

Anti-*P. aeruginosa* antibodies. The results from the anti-*P. aeruginosa* IgA, IgG, and IgM antibody assays are illustrated in Fig. 1. IPP immunization with *P. aeruginosa* induced a strong antibody response both in the sera (IgA, IgG, and IgM) and in the bronchoalveolar secretions (IgA and IgG). In both cases, IgA was the most significantly increased isotype. The increase in bronchoalveolar IgM from immune animals failed to reach significance ($P = 0.079$ compared with that in nonimmune animals).

Phagocytic indices. Light microscopy evaluation of cytospin preparations allowed easy identification of PMN that had phagocytosed bacteria (Fig. 2). Intracellular localization of the phagocytosed bacteria was confirmed with transmission electron microscopy (Fig. 2). After both 0.5 and 4 h of challenge, pulmonary PMN that had phagocytosed more than five bacteria were found in significantly greater

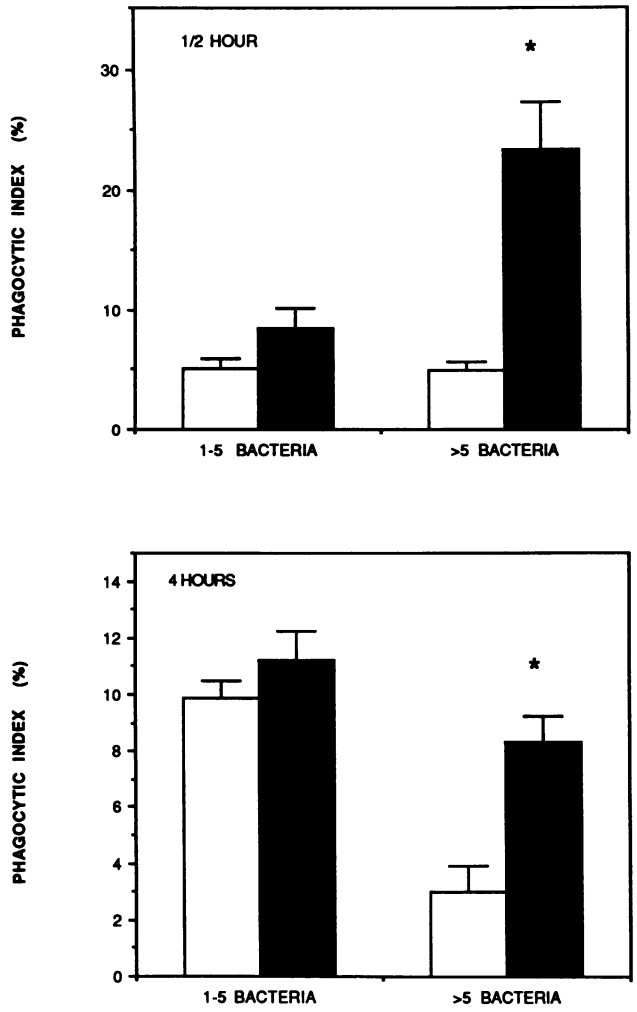

FIG. 3. Phagocytic indices in bronchoalveolar PMN from immune (■) and nonimmune (□) rats challenged for 0.5 or 4 h with live *P. aeruginosa* cells. The figure shows the percentages of PMN that have phagocytosed 1 to 5 or >5 bacteria for each group. Values are $\bar{x}$s ± SEMs from 15 immune and 22 nonimmune animals. *, $P < 0.01$ compared with values for nonimmune animals.

concentrations in the lavages from immune rats than in those of nonimmune animals, whereas the percentages of PMN that had phagocytosed one to five bacteria were not significantly different between both groups (Fig. 3).

Chemiluminescence. In all experiments, PMN populations isolated on Percoll were >95% viable and >98% pure. The results from the PMN chemiluminescence measurements are illustrated in Fig. 4. In the presence of zymosan opsonized with nonimmune serum, PMN from both nonimmune (*NI*) and immune (*I*) rats reached peak chemiluminescence at 5 min (i.e., $NI = 64.9 \pm 31.2$; $I = 215.0 \pm 42.2$). Similar values were obtained for peak chemiluminescence when zymosan was opsonized with immune serum (i.e., $NI = 58.3 \pm 29.8$ at 7 min; $I = 201.5 \pm 40.0$ at 5 min). In PMN from immune rats, chemiluminescence at 2 and 5 min was significantly ($P < 0.05$) greater than that in PMN from nonimmune animals. The type of serum used for opsonization (i.e., from immune or nonimmune animals) did not seem to affect the chemiluminescence response of either PMN population (Fig. 4).

PMN function. The results from the PMN function assays are summarized in Table 2. Compared with PMN from nonimmune rats, PMN from immune animals exhibited significantly ($P < 0.05$) enhanced chemotaxis for casein and, even more significantly ($P < 0.005$), enhanced chemotaxis for live *P. aeruginosa*. Chemokinesis in the presence of either type of serum was significantly ($P < 0.005$) increased in PMN from immune rats. No significant difference between the PMN random migration rates of both groups was observed.

LTB_4 synthesis. Levels of LTB_4 in serum samples, bronchoalveolar lavages, and lung homogenates are illustrated in Fig. 5. After 0.5 h of challenge, LTB_4 levels between both groups were not different in any of the areas studied. After 4 h, no difference had appeared in the serum samples. However, at 4 h, the LTB_4 content in bronchoalveolar lavages from nonimmune rats was increased 20-fold over values from immune or control animals, which were not different. Also at 4 h, LTB_4 levels in lung homogenates of both the nonimmune and the immune groups were significantly ($P < 0.05$) increased over those in controls.

DISCUSSION

This study correlates enhancement of pulmonary PMN recruitment, activity, and function with acceleration of *P. aeruginosa* clearance from the lungs of rats intestinally immunized with killed bacteria. Priming of the gut-associated lymphoid tissue with killed mucoid *P. aeruginosa* cells induced protection against live pulmonary challenge with the homologous strain. After pulmonary challenge with live *P. aeruginosa* cells, compared with phagocytes from nonimmune sham-treated rats, bronchoalveolar PMN from immunized animals were found in significantly greater numbers and had phagocytosed more bacteria. These changes were associated with increased bacterial clearance and enhanced PMN chemiluminescence, chemokinesis, and specific chemotaxis but not with increased levels of LTB_4 in the bronchoalveolar lavages. The results indicate that IPP immunization, which mimics intestinal immunization (7, 18), can favorably alter the course of a *P. aeruginosa* infection in the lung and that accelerated recruitment and increased activation of bronchoalveolar PMN are important effector mechanisms of protective immunity in the present model. Enhanced LTB_4 synthesis in the alveolar space does not appear to be implicated in this response.

Consistent with the hypothesis that cellular effector mechanisms play a central role in the protection of the immune lung, subsequent pulmonary challenge in mice immunized with *Listeria monocytogenes* is controlled by an influx of activated macrophages (46). In addition, experiments performed in this laboratory with an animal model similar to the one described here identified PMN activation as an effector mechanism of protective immunity against nontypeable *Haemophilus influenzae* after priming of the gut-associated lymphoid tissue (47). In addition, studies of mice immunized against *P. aeruginosa* with a mixture of vinblastine sulfate and a *P. aeruginosa* polysaccharide have shown that this microorganism could be directly killed by T lymphocytes after an intraperitoneal challenge (24, 34). The authors did not assess whether PMN were implicated in protective immunity in vivo. Indeed, the present study demonstrates that one of the mechanisms which contributes to accelerated *P. aeruginosa* clearance from the lungs of immunized rats is an enhancement of the pulmonary PMN response to the pathogen. Further investigations will clarify the respective

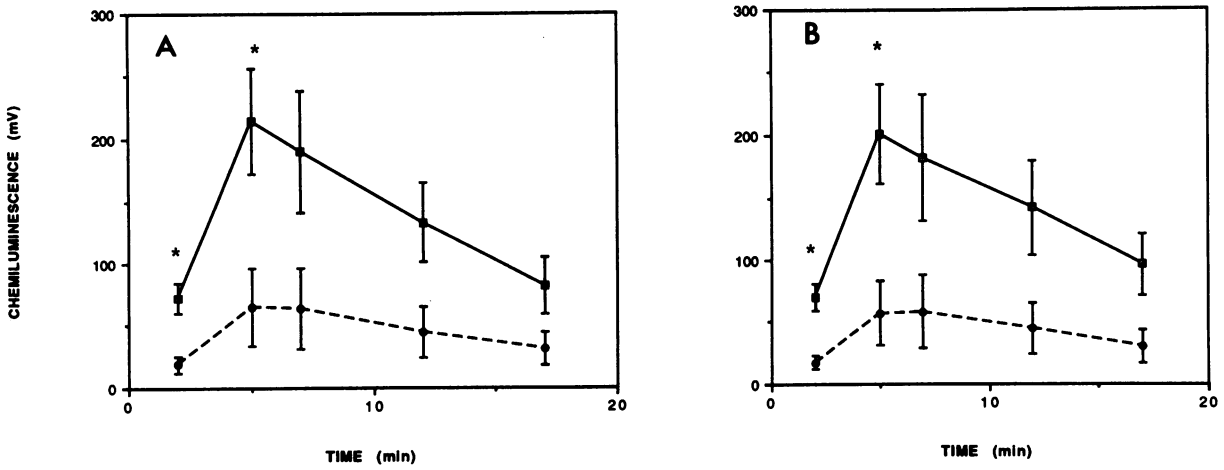

FIG. 4. Luminol-enhanced chemiluminescence of PMN purified from the bronchoalveolar lavages of immune (⊡) and nonimmune (●) rats after 4 h of challenge with live *P. aeruginosa* cells. Measurements were taken in the presence of zymosan opsonized with pooled serum samples from nonimmune (A) or immune (B) animals. Values are $\bar{x}$s ± SEMs from 10 immune animals and from five cell pools of 22 nonimmune animals. *, $P < 0.05$ compared with values for nonimmune animals.

roles played by T cells and PMN in pulmonary immunity to *P. aeruginosa*.

The results from the phagocytic-index studies revealed an increase in the percentage of PMN that had phagocytosed >5 bacteria in immune animals. In contrast, the concentrations of PMN that had phagocytosed one to five bacteria were not different between the PMN populations of immune and nonimmune animals, suggesting that immunization had enhanced the phagocytic activity of individual PMN. Moreover, PMN from immune animals exhibited a more significantly increased chemotaxis for live *P. aeruginosa* ($P < 0.005$) than for casein ($P < 0.05$), compared with PMN from nonimmune rats. Further experiments will allow a determination of whether the findings presented here reflect a specific enhancement of the PMN immune response to *P. aeruginosa* or whether immunization increases PMN sensitivity to *P. aeruginosa* chemotaxins, which seem to be active even in the absence of host humoral factors (11).

The protective role of pulmonary PMN against a primary *P. aeruginosa* infection has been well documented. In immunosuppressed guinea pigs, increased mortality following a pulmonary challenge with *P. aeruginosa* correlated with a diminished PMN response in the lung (31). In another study, granulocyte transfusion therapy favorably altered the course of experimental *P. aeruginosa* pneumonia in leukopenic dogs (3). More recently, unsuccessful pulmonary clearance of the microorganism in young mice has been associated with a transient, age-dependent reduction in PMN activity (42). Hence, in addition to the obvious significance of the role played by PMN in controlling primary infections with *P. aeruginosa*, the present study now shows that PMN activation is a central effector mechanism of pulmonary immunity following gut-associated lymphoid tissue priming. In addition, since IPP immunization in this model provides protection in the lung, these findings further support the concept of a common mucosal system (27). Finally, since it appears that mucoid and nonmucoid *P. aeruginosa* variants are equally sensitive to killing by a 55-kDa bactericidal protein from human PMN (41), the results may identify enhanced pulmonary PMN function as a possible therapeutic target for the development of an effective anti-*P. aeruginosa* pneumonia vaccine. However, because PMN have also been implicated in the propagation of lung injury in patients with CF (28) as well as in damage to other mucosal systems (49, 50), possible detrimental side effects due to immunization-induced PMN activation must be carefully investigated.

The apparent efficacy of the role played by PMN in controlling primary and secondary pulmonary infections with *P. aeruginosa* may seem surprising in light of the numerous immunosuppressive activities displayed by this microorganism. Indeed, it has been established that *P. aeruginosa* may impair opsonic phagocytosis via the proteolysis of IgG and C3b opsonic molecules (9, 10, 39, 45), via the barrier effect of alginate preventing antibody and/or complement binding (25, 26), and/or via the breakdown of opsonic fibronectin (51). In addition, leucocidin, exotoxin A, hemolysin, and a 65-kDa PMN inhibitor, all released by *P. aeruginosa*, as well as the inactivation of interleukin-2,

TABLE 2. Functions of pulmonary PMN from nonimmune and immune rats following a 4-h challenge with live *P. aeruginosa*[a]

Rat group	Random migration (μm/h)	Chemotaxis (μm/h)		Chemokinesis (μm/h)	
		Casein	Live *P. aeruginosa*	Nonimmune serum	Immune serum
Nonimmune	2.0 ± 0.4	44.9 ± 4.2	5.1 ± 1.1	16.1 ± 2.9	21.2 ± 5.2
Immune	1.8 ± 0.6	75.1 ± 10.0	32.5 ± 5.9	56.2 ± 3.2	61.8 ± 7.0
P	>0.8	<0.05	<0.005	<0.005	<0.005

[a] $\bar{x}$ and SEM values are from five and seven values, respectively. Each value for the nonimmune group was calculated from five different cell pools obtained from four to nine animals.

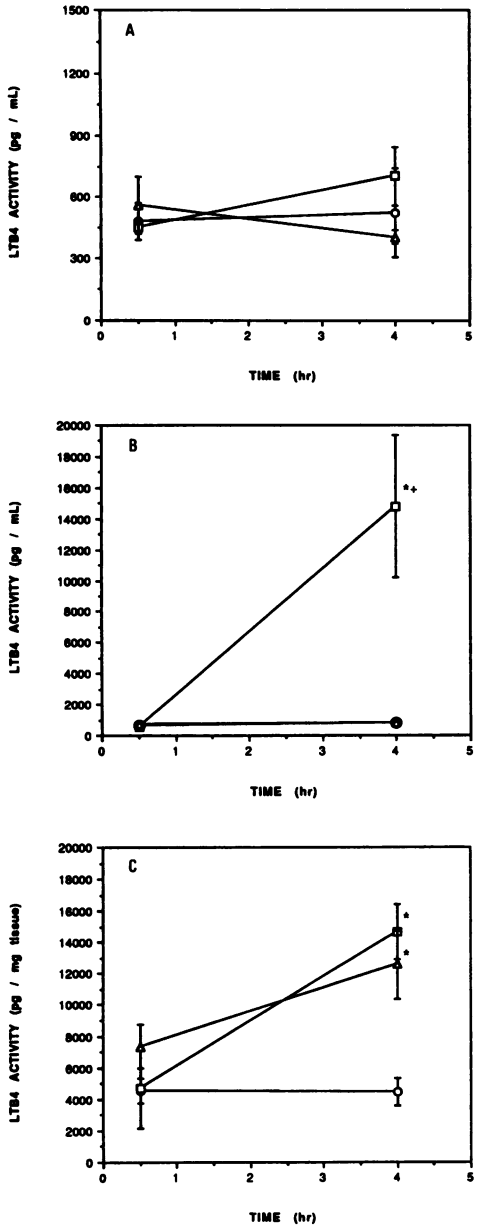

FIG. 5. LTB_4 synthesis in control (○), nonimmune (□), and immune (△) rats. Measurements were taken from sera (A), bronchoalveolar lavages (B), and pulmonary tissue homogenates (C) after 0.5 or 4 h of challenge with live *P. aeruginosa* cells. Values are $\bar{x}$s ± SEMs from three control, five nonimmune, and five immune animals. *, $P < 0.05$ compared with values for control animals; +, $P < 0.05$ compared with values for immune animals.

gamma interferon, and tumor necrosis factor alpha, may all further contribute to the inhibition of cell-mediated immunity (16, 20, 29, 30, 37). Yet in the present study, after 0.5 h of challenge, immune animals already displayed a significantly increased recruitment of bronchoalveolar PMN over that with nonimmune animals, while immunization alone, i.e., which was not followed by a pulmonary challenge, had no effect on PMN recruitment into the lungs. As PMN recruitment, function, and activity increased, the rate of bacterial clearance was gradually accelerated in immune animals. In addition, the calculated ratio of the number of cleared bacteria to alveolar PMN in immune animals (~8:1) fits well within the physiological range of the numbers of phagocytosed bacteria counted in PMN from immune animals (1 to >20 [Fig. 2]). These results unequivocally demonstrate that activation of pulmonary PMN is an effective component of the immune defense against *P. aeruginosa* and suggest that the host may override bacterial immunosuppression via mechanisms which are not yet defined.

Upon artificial or natural stimulation, leukocytes such as PMN or macrophages and other cell types can produce LTB_4 via the 5-lipoxygenase pathway. This eicosanoid is one of the most potent chemoattractants for PMN. LTB_4-mediated neutrophil recruitment has been reported in various models of mucosal inflammation (17, 40, 48, 50). Studies of the generation of eicosanoids in human blood after the phagocytosis of zymosan showed that LTB_4 synthesis increased to maximum levels from 30 to 60 min after the stimulus and remained essentially constant thereafter for at least another hour (12). Also, increased LTB_4 release by PMN parallels the kinetics of opsonic phagocytosis of *H. influenzae* in vitro (14). Finally, in an animal model of colonic inflammation, increased LTB_4 synthesis correlated with PMN infiltration for a duration of several weeks (50). In keeping with these observations, the present study showed that PMN infiltration was associated with markedly increased LTB_4 synthesis in the bronchoalveolar spaces and pulmonary tissues of nonimmune rats acutely challenged with mucoid *P. aeruginosa*. In contrast, the data clearly demonstrate that LTB_4 production does not explain the enhanced PMN recruitment and subsequent clearance in rats previously immunized with mucoid *P. aeruginosa*. In conclusion, these findings implicate LTB_4 as an important amplifier of the inflammatory response during a primary pulmonary infection with mucoid *P. aeruginosa*; however, following immunization and subsequent live pulmonary challenge with this microorganism, accelerated PMN infiltration into the alveolar space is not LTB_4 dependent. The mechanisms circumventing LTB_4 synthesis (or, alternatively, inactivating free LTB_4) in this model need to be clarified, but this observation alone represents a promising finding in light of the detrimental effects of LTB_4 in a number of mucosal diseases. We are currently investigating whether enhanced interleukin-1 production may explain LTB_4-independent PMN recruitment and activation in the immune lung.

In summary, the findings from the present study show that enhancement of the PMN response to an infection challenge represents a central effector mechanism of protective immunity against mucoid *P. aeruginosa* when a host is intestinally immunized against this pathogen. Since the results presented here suggest that this response is not associated with LTB_4 synthesis in the alveolar space, the mediators of the immune cascade leading to PMN recruitment and activation during a *P. aeruginosa* infection challenge have yet to be defined. Further studies following this direction will shed more light on the immune processes relevant to oral immunization against *P. aeruginosa* infections of the respiratory tract and may lead to the development of combined therapeutic procedures involving vaccination and the use of immunogenic regulators.

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