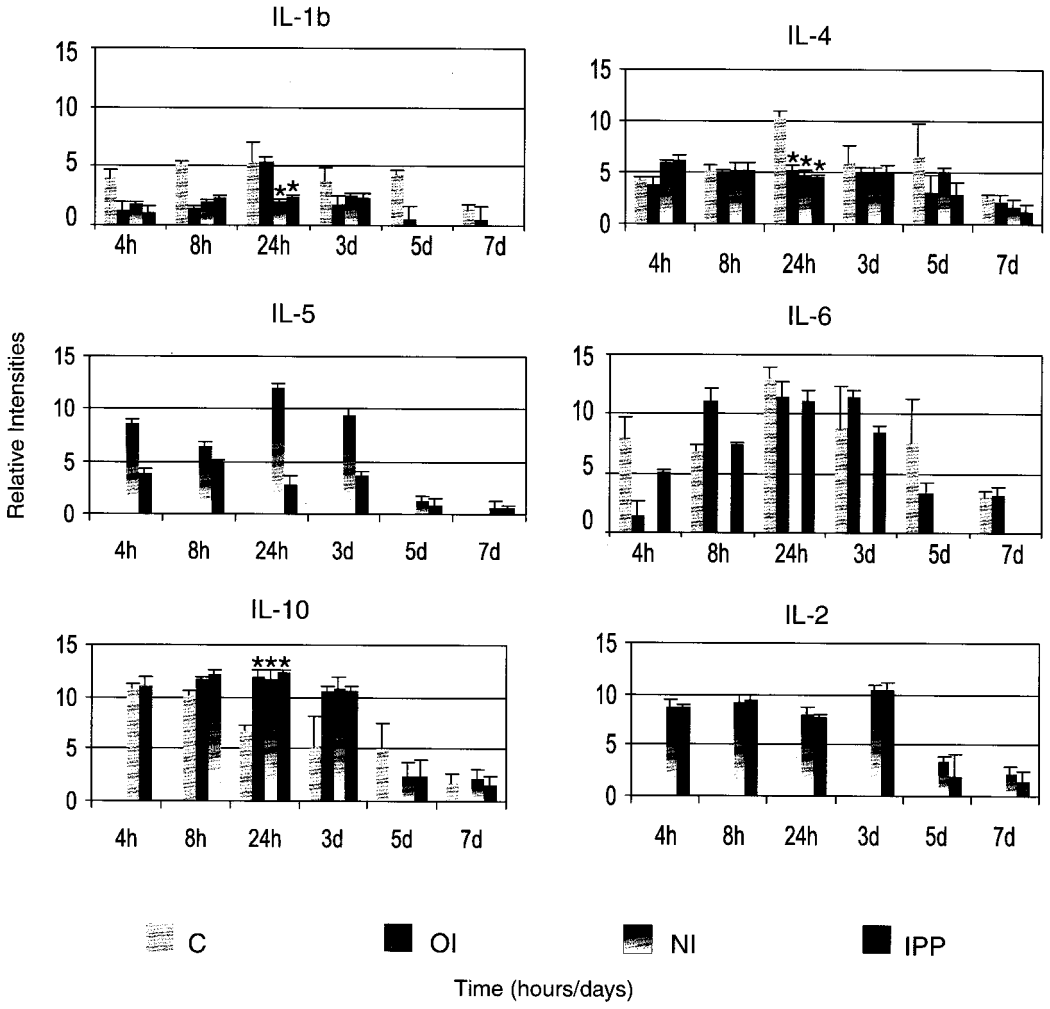

FIG. 7. Kinetics and levels of cytokine mRNA expression quantified by RNase protection assay in whole rat eyes of nonimmunized (C) and immunized rats challenged with cytotoxic *P. aeruginosa* strain 6206. The graphs show means ± SEMs from three experiments, with five animals in each group at each time point, normalized with two housekeeping genes (L32 and GAPDH). Mean differences were considered significant (*) when P was ≤ 0.05. OI, orally immunized; NI, nasally immunized; IPP, IPP immunized.

DISCUSSION

Our study showed that the route of immunization affects the severity and persistence of microbial keratitis. Immunization has the potential to modulate the inflammatory response to an infection. This modulation includes the production of chemical signals, cytokines and chemokines, with recruitment and activation of cells involved in clearing the infection (29). This study has demonstrated that immunization changes the kinetics of PMN infiltration, with immune groups having more rapid recruitment and resolution of PMNs in the cornea than the nonimmune group. Associated with this was a more rapid clearance of bacteria, differences in the levels of cytokines expressed and produced, and reduced adverse pathology. In particular, the IPP and intranasal immunization regimes with an OT boost provided the best protection from corneal ulceration.

CINC-1 is a potent activator and attractant of neutrophils (27). Increased CINC-1 levels were detected earlier (4 to 8 h) postinfection in immunized rats than in nonimmunized rats, with all groups peaking at 24 h postchallenge. However, despite the earlier increased production of CINC-1 in the immunized groups, the peak levels of CINC-1 were significantly lower in the immunized groups and also decreased far more rapidly. The changes in the CINC-1 levels corresponded to the recruitment and resolution profiles of the PMNs. The rate of PMN recruitment in other disease settings has been associated with early bacterial clearance, such as enhanced respiratory clearance of nontypeable *Haemophilus influenzae* following mucosal immunization (5, 10). Persistence of PMNs in the nonimmune animals during the later stages of infection may contribute to corneal scarring and perforation.

For the PMN response to be beneficial rather than detrimental, a rapid resolution of PMN infiltration must occur. Immunization of rats against *P. aeruginosa* corneal infection achieved a rapid resolution of PMN infiltrates. In addition to the modulation of CINC-1 levels, there were reduced levels of the proinflammatory cytokines (IL-1β and IL-6) and similar or higher levels of the cytokines associated with immunosuppres-

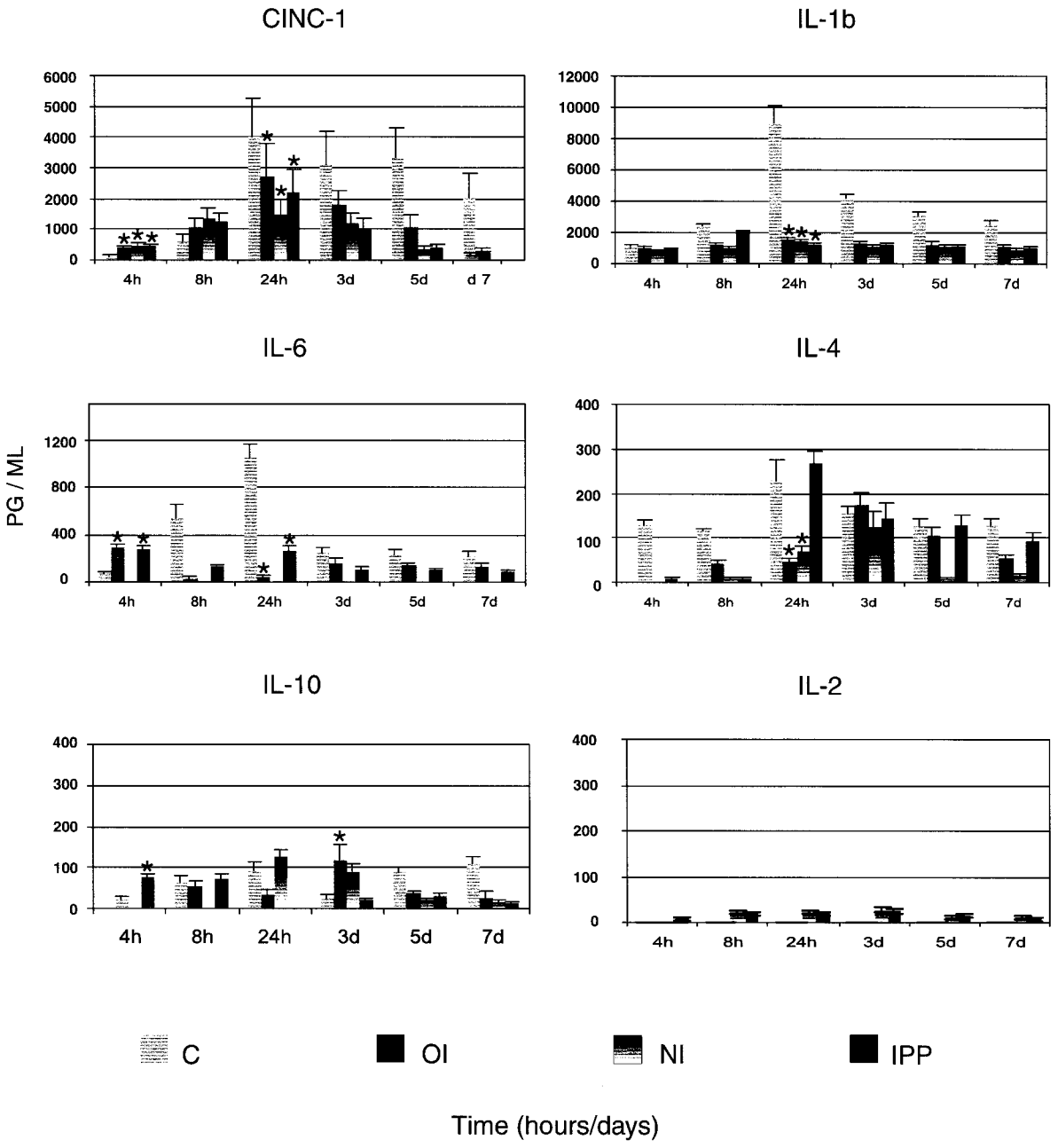

FIG. 8. Kinetics and levels of cytokine protein secretion quantified by ELISA in whole rat eyes of nonimmunized (C) and immunized rats challenged with cytotoxic *P. aeruginosa* strain 6206. The results are presented as means ± SEMs from three experiments, with three animals in each group at each time point. Mean differences were considered significant (∗) when P was ≤0.05.

sive or IgA antibody responses (IL-10 and IL-4). Balanced expression of proinflammatory and anti-inflammatory cytokines in the immunized animals compared to the overwhelming proinflammatory cytokine response in the nonimmune group may control inflammation by regulating not only inflammatory cell recruitment but also IgA secretion.

Clearance of bacteria from the ocular surface is presumed to involve the combined actions of PMNs and secretory IgA. Immunization induced significantly elevated levels of antigen-specific IgA in tears and IgG in serum. The role of antigen-specific antibodies in protection against corneal infection is controversial, with correlation between the presence of antibody and protection not always being clearly defined. A recent (24) study has shown that secretory IgA can significantly inhibit *P. aeruginosa* binding to wounded mouse cornea in vitro, thereby protecting against keratitis. One of the mechanisms by which IgA antibodies may prevent bacterial colonization is by specifically interacting with bacterial adhesins required for binding to mucosal tissue (24). IgA is capable of potentiating the function of innate antibacterial factors and interacting with mucosal phagocytic cells and lymphocytes (25). Oral immunization with *Acanthamoeba* spp. antigen mixed with cholera

toxin induces the production of parasite-specific IgA in mucosal secretions and prevents corneal infection (23). Although antigen-specific IgG antibody appears to be important for opsonophagocytosis (34), a correlation between the presence of opsonizing antibodies and protection in vivo has not been clearly determined to be an essential mechanism of effective immunity (33, 35). There is also evidence that suggests that systemically derived IgG may also be capable of conferring protection in the cornea (28). In addition to measuring significant titers of antigen-specific IgA in tears, we have demonstrated the presence of a group of IgA-enhancing Th2-type cytokines (IL-4, IL-5, IL-6, and IL-10) which may provide an environment for preferential immunoglobulin class switching for IgA in the eye.

Previous studies using a rat model for pulmonary *P. aeruginosa* infection have shown that mucosal immunization significantly alters the profile of inflammatory cytokines produced in response to infection (5). Other evidence also suggests that nasal and IPP immunization with mucosal adjuvant induces dominant Th2 responses in nasal-associated lymphoid tissue and Peyer's patches (12, 39). This study has shown that the route of immunization changes the profile of cytokine expression during *P. aeruginosa* corneal infection, with the most significant differences appearing in the nasal and IPP immunization groups. Expression of IL-2 and IL-5 were especially altered, with nasally immunized rats expressing high levels of IL-5 and baseline levels of IL-6 mRNA, with corresponding baseline levels of IL-6 protein. In contrast, orally immunized rats showed no IL-5 expression but had high IL-6 expression and secretion, while IPP immunization resulted in the upregulation of both IL-5 and IL-6. IL-5 and IL-6 are known to differentially influence the B-1 and B-2 lineage of plasma cells (2). Collectively, the data suggest that nasally immunized animals may be producing IgA plasma cells of B-1 lineage, which are IL-5 dependent and IL-6 independent (2), whereas orally immunized animals may be producing predominantly cells of B-2 lineage. B-1 cells are physically and functionally unique B cells producing antibodies to bacterial antigens such as lipopolysaccharide and phosphocholine (1). B-1 cells mainly reside in mucosal effector tissues, while conventional IgA^+ B-2 cells reside in mucosal inductive sites (39). Nasal-associated lymphoid tissue functions as a primary inductive site for IgA antibody in tears by contributing triggered IgA-committed B cells to the lacrimal gland (22). A recent study (30) has shown that a high frequency of IgA-committed B-1 cells occurs in the lacrimal gland (an effector site).

A role for T cells and cytokines produced by activated T cells in protection from ocular bacterial infections has not been demonstrated previously. Nasally and IPP-immunized rats induced antigen-specific lymphocyte responses, providing evidence that an antigen-specific T-lymphocyte response was induced by immunization and that these lymphocytes migrated from the site of immunization. Immunologically specific T cells recruit neutrophils in an antigen-dependent and dose-dependent fashion (6). Cytokines released by activated T cells may direct the activity of nonspecific effector cells (21, 37). All of these studies have shown the involvement of T cells and cytokines in respiratory disease models. Evidence that supports the relevance of a $CD4^+$ Th1- versus Th2-type immune response was presented in a study that used a mouse *P. aeruginosa* keratitis model. Data from this study suggest that Th2-responsive mice regulate inflammatory cellular infiltrates more efficiently by downregulating the inflammatory response, which in turn results in less corneal stromal damage (11, 20). Further studies are required to define the importance of a T-cell response in protection against ocular infection.

This study has demonstrated that the immunization route modulates the inflammatory response to ocular *P. aeruginosa* infection, thus affecting the severity of keratitis and adverse pathology. The results show that immunization affects the rate of bacterial clearance and alters the profile of cytokines produced in response to ocular infection, with nasal immunization resulting in the most significant level of protection. The results suggest that the degree of protection afforded by immunization may depend upon the rapid recruitment of PMNs, the induction of antigen-specific IgA, and the balanced production of proinflammatory and immunosuppressive cytokines and that T-cell responses may influence these events.

ACKNOWLEDGMENTS

This research was partly supported by the Australian Federal Government through the Cooperative Research Centres Program.

We thank Reg Wong for excellent statistical analysis, Wen Wang for technical assistance, Denise Lawler and Robyn Lawler for helping with animal experiments, and Philip Julian and Carol Woollcott for their help in preparing illustrations.

REFERENCES

1. **Aramaki, M., T. Nagasawa, T. Koeshi, and I. Ishikawa.** 1998. Presence of activated B-1 cells in chronic inflamed gingival tissue. J. Clin. Immunol. **18:**421–429.
2. **Bao, S., K. W. Beagley, A. M. Murray, V. Caristo, K. I. Matthaei, I. G. Young, and A. J. Husband.** 1998. Intestinal IgA plasma cells of the B1 lineage are IL-5 dependent. Immunology **94:**181–188.
3. **Beagley, K. W., J. H. Eldridge, H. Kiyono, and F. Lee.** 1989. Interleukins and IgA synthesis: human and murine IL-6 induce high rate of IgA secretion in IgA-committed B-cells. J. Exp. Med. **169:**2133–2148.
4. **Bradley, P. P., R. D. Christensen, and G. Rothstein.** 1982. Cellular and extracellular myeloperoxidase in pyogenic inflammation. Blood **60:**618–625.
5. **Buret, A., M. L. Dunkley, G. Pang, R. L. Clancy, and A. W. Cripps.** 1994. Pulmonary immunity to *Pseudomonas aeruginosa* in intestinally immunized rats: role of alveolar macrophages, tumor necrosis factor, and interleukin-la. Infect. Immun. **62:**5335–5343.
6. **Campbell, P. A.** 1990. The neutrophil, a professional killer of bacteria, may be controlled by T cells. Clin. Exp. Immunol. **79:**141–143.
7. **Challacombe, S. J., and T. B. Tomasi, Jr.** 1980. Systemic tolerance and secretory immunity after oral immunization. J. Exp. Med. **152:**1459–1472.
8. **Cole, N., M. D. P. Willcox, S. M. J. Fleiszig, F. Stapleton, S. Bao, S. Tout, and A. Husband.** 1998. Different strains of *Pseudomonas aeruginosa* isolated from ocular infections or inflammation display distinct corneal pathologies in an animal model. Curr. Eye Res. **17:**730–735.
9. **Cripps, A. W., M. L. Dunkley, and R. L. Clancy.** 1994. Mucosal and systemic immunizations with killed *Pseudomonas aeruginosa* protect against acute respiratory infection in rats. Infect. Immun. **62:**1427–1436.
10. **Foxwell, A. R. C., J. M. Kyd, and A. W. Cripps.** 1998. Characterization of the immunological response in the clearance of nontypable Haemophilus influenzae from the lung. Immunol. Cell Biol. **76:**323–331.
11. **Hazlett, L. D., S. McClellan, B. Kwon, and R. Barrett.** 2000. Increased severity of *Pseudomonas aeruginosa* corneal infection in strains of mice designated as Th1 versus Th2 responsive. J. Immunol. **41:**805–810.
12. **Hiroi, T., M. Yanagita, H. Iijima, K. Iwatani, T. Yoshida, K. Takatsu, and H. Kiyono.** 1999. Deficiency of IL-5 receptor a-chain selectively influences the development of the common mucosal immune system independent IgA-producing B-1 cells in mucosa-associated tissue. J. Immunol. **162:**821–828.
13. **Hobden, J. A., S. A. Masinick, R. P. Barrett, and L. D. Hazlett.** 1997. Proinflammatory cytokine deficiency and pathogenesis of *Pseudomonas aeruginosa* keratitis in aged mice. Infect. Immun. **65:**2754–2758.
14. **Inoue, Y., Y. Shimomura, and R. Manabe.** 1992. Herpes simplex virus glycoprotein D: protective immunity against murine herpetic keratitis. Investig. Ophthalmol. Vis. Sci. **31:**411–418.
15. **Kelleher, R. S., L. E. Hann, and J. A. Edwards.** 1991. Endocrine, neural and immune control of secretory component output by lacrimal gland acinar cells. J. Immunol. **146:**3405–3412.

16. **Kernacki, K. A., and R. S. Berk.** 1994. Characterization of the inflammatory response induced by corneal infection induced by *Pseudomonas aeruginosa*. J. Ocul. Pharmacol. **10:**281–288.
17. **Kernacki, K. A., J. A. Hobden, L. D. Hazlett, R. Friedman, and R. S. Berk.** 1995. *In vivo* bacterial protease production during *Pseudomonas aeruginosa* corneal infection. Investig. Ophthalmol. Vis. Sci. **36:**1371–1379.
18. **Kernacki, K. A., R. P. Barrett, J. A. Hobden, and L. D. Hazlett.** 2000. Macrophage inflammatory protein-2 is a mediator of polymorphonuclear neutrophil influx in ocular bacterial infection. J. Immunol. **164:**1037–1045.
19. **Kreger, A. S., D. M. Lyerly, L. D. Hazlett, and R. S. Berk.** 1986. Immunization against experimental *Pseudomonas aeruginosa* and *Serratia marcescens* keratitis. Vaccination with lipopolysaccharide endotoxins and proteases. Investig. Ophthalmol. Vis. Sci. **27:**932–939.
20. **Kwon, B., and L. D. Hazlett.** 1997. Association of $CD4^+$ T cell-dependent keratitis with genetic susceptibility to *Pseudomonas aeruginosa* ocular infection. J. Immunol. **159:**6283–6290.
21. **Kyd, J. M., M. L. Dunkley, and A. W. Cripps.** 1995. Enhanced respiratory clearance of nontypable *Haemophilus influenzae* following mucosal immunization with P6 in rat model. Infect. Immun. **63:**2931–2940.
22. **Lather, D. M. R., R. F. Gill, and P. C. Montgomery.** 1998. Inductive pathways leading to rat tear IgA antibody responses. Investig. Ophthalmol. Vis. Sci. **39:**1005–1011.
23. **Leher, H. F., H. Alizadeh, W. M. Taylor, A. S. Shea, R. S. Silvany, F. V. Klink, M. J. Jager, and J. Y. Niederkorn.** 1998. Role of mucosal IgA in the resistance to *Acanthamoeba* keratitis. Investig. Ophthalmol. Vis. Sci. **39:** 2666–2673.
24. **Masinick, S. A., C. P. Montgomery, P. C. Montgomery, and L. D. Hazlett.** 1997. Secretory IgA inhibits *Pseudomonas aeruginosa* binding to cornea and protects against keratitis. Investig. Ophthalmol. Vis. Sci. **38:**910–918.
25. **McGhee, J. R., and H. Kiyono.** 1993. New perspective in vaccine development: mucosal immunity to infections. Infect. Agents Dis. **2:**55–73.
26. **Moon, M. M., L. D. Hazlett, R. E. Hancock, R. S. Berk, and R. Barrett.** 1988. Monoclonal antibodies provided protection against ocular *Pseudomonas aeruginosa* infection. Investig. Ophthalmol. Vis. Sci. **29:**1277–1284.
27. **Mulligan, M. S., M. L. Jones, M. A. Bolanowski, M. P. Baganoff, W. L. Deppeler, D. M. Meyers, U. S. Ryan, and P. A. Ward.** 1993. Inhibition of lung inflammatory reactions in rats by an anti-human IL-8 antibody. J. Immunol. **150:**5585–5595.
28. **Preston, M. J., A. A. Gerecker, N. L. Koles, M. Pollack, and G. B. Pier.** 1997. Prophylactic and therapeutic efficacy of immunoglobulin G antibodies to *Pseudomonas aeruginosa* lipopolysaccharide against murine experimental corneal infection. Investig. Ophthalmol. Vis. Sci. **38:**1418–1425.
29. **Rudner, X. L., K. A. Kernacki, R. P. Barrett, and L. D. Hazlett.** 2000. Prolonged elevation of IL-1 in *Pseudomonas aeruginosa* ocular infection regulates macrophage-inflammatory protein-2 production, polymorphonuclear neutrophil persistence, and corneal perforation. J. Immunol. **164:**6576–6582.
30. **Saitoh-Inagawa, W., H. Takachika, M. Yanagita, H. Iijima, E. Uchio, S. Ohno, K. Aoki, and H. Kiyono.** 2000. Unique characteristics of lacrimal gland as a part of mucosal immune network: high frequency of IgA-committed B-1 cells and NK1.1+ ab T cells. Investig. Ophthalmol. Vis. Sci. **41:**138–144.
31. **Schein, O. D., L. D. Ormerod, and E. Barraquer.** 1989. Microbiology of contact lens associated keratitis. Cornea **8:**281–285.
32. **Steuhl, K. P., G. Doring, and H. J. Thiel.** 1989. The significance of bacterial and host factors in corneal infections caused by *Pseudomonas aeruginosa*. Fortschr. Ophthalmol. **86:**283–286.
33. **Troelstra, A., L. Vogel, L. van Alphen, P. Eijk, H. Jansen, and J. Dankert.** 1994. Opsonic antibodies to outer membrane protein P2 of nonencapsulated *Haemophilus influenzae* are strain specific. Infect. Immun. **62:**779–784.
34. **Verhoef, J., and M. R. Visser.** 1993. Neutrophil phagocytosis and killing: normal function and microbial evasion, p. 108–137. *In* J. S. Abramson and J. G. Wheeler (ed.), The neutrophil. Oxford University Press, New York, N.Y.
35. **Wallace, F. J., R. L. Clancy, and A. W. Cripps.** 1989. An animal model demonstration of enhanced clearance of non-typable *Haemophilus influenzae* from respiratory tract after antigen stimulation of gut associated lymphoid tissue. Am. Rev. Respir. Dis. **140:**311–316.
36. **Wallace, F. J., A. W. Cripps, R. L. Clancy, A. J. Husband, and C. S. Witt.** 1991. A role for intestinal T lymphocytes in bronchus mucosal immunity. Immunology **74:**68–73.
37. **Wallace, F. J., C. S. Witt, R. L. Clancy, A. W. Cripps, and A. J. Husband.** 1995. Protection against non-typable *Haemophilus influenzae* following sensitization of gut lymphoid tissue: role of specific antibody and phagocytes. Immunol. Cell Biol. **73:**258–265.
38. **Welsh, N. H., A. J. Rauch, and S. L. Graffin.** 1984. Topical immunotherapy for *Pseudomonas* keratitis in rabbits: use of anti-lipopolysaccharide plasma. Br. J. Ophthalmol. **68:**828–832.
39. **Yanagita, M., T. Hiroi, N. Kitagaki, S. Hamada, H. Ito, H. Shimauchi, H. Murakami, and H. Kiyono.** 1999. NALT immunity: fimbriae-specific Th1 and Th2 cell regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokines. J. Immunol. **162:**3559–3565.

Editor: J. D. Clements