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Analysis of Protective and Nonprotective Monoclonal Antibodies Specific for *Bordetella pertussis* Lipooligosaccharide

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In this study, it has been determined that immunoglobulin G1 (IgG1) and IgG3 monoclonal antibodies directed to the lipooligosaccharide A of *Bordetella pertussis* were able to protect mice from fatal aerosol infection. No correlation was found between the bactericidal activity in vitro in the presence of complement and the protection in mice, since a bactericidal IgG3 did not elicit protection. In addition, no significant difference in protective capacity was observed with bactericidal and nonbactericidal IgG1 antibodies, indicating that bactericidal activity is not a requirement for protection mediated by certain anti-lipooligosaccharide A antibodies. A reduction in protection in C5-deficient mice was observed, suggesting a significant role for complement in certain host defense mechanisms against *B. pertussis* infection.

Although widespread use of a whole-cell pertussis vaccine has been effective in protection against clinical disease (8, 10), the mechanism by which vaccination leads to protective immunity to pertussis is currently not understood. High titers of vaccine-induced bacterial agglutinins have historically been correlated with the clinical efficacy of whole-cell pertussis vaccines (8). One component of this vaccine that induces serum agglutinins is the carbohydrate moiety of *Bordetella pertussis* lipooligosaccharide A (LOS A) (12).

B. pertussis LOS lacks a long-chain polysaccharide O antigen and is resolved electrophoretically into two bands, a slowly migrating band designated LOS A and a fast-migrating band designated LOS B (14). These two species differ in molecular weight as three *N*-acetyl amino sugars found on LOS A are not present on LOS B (5). Murine and human monoclonal antibodies to the carbohydrate portion of LOS A have been shown to be highly specific for *B. pertussis*, and to be bactericidal in vitro (1, 2, 12, 13). Mountzouros et al. have found that one particular monoclonal antibody specific for LOS A that is bactericidal in vitro is effective in reducing colonization in a mouse model of *B. pertussis* respiratory infection (13).

The availability of multiple monoclonal antibodies specific for *B. pertussis* LOS A provides an opportunity to study mechanisms involved in protective antibody-mediated immunity. We have analyzed the protective capacity of five monoclonal antibodies specific for the LOS A component of *B. pertussis* that differ in their in vitro bactericidal capacity (1). This paper is the first description of the in vivo function of these monoclonal antibodies. We have also analyzed the ability of a protective monoclonal antibody to protect mice deficient in the classical pathway of complement.

BL-1, BL-6, and BL-7 are mouse immunoglobulin G1 (IgG1) monoclonal antibodies to LOS A (1) and were purified on protein G-Sepharose (Pharmacia LKB Technology, Piscataway, N.J.). BL-2 and BL-5 are IgG3 anti-LOS A monoclonal antibodies and were purified by low-salt precipitation (11). P2-3 (IgG3), P2-12 (IgG1), and 11-B9 (IgG1) are monoclonal antibodies that were used as isotype-matched negative controls and were purified as described above. Purified monoclonal antibodies were analyzed for total protein content (Bio-Rad, Richmond, Calif.) and filter sterilized. Each preparation of purified monoclonal anti-LOS A antibody was tested for in vitro bactericidal activity and for antibody titer by enzyme-linked immunosorbent assay (ELISA) as previously described (1).

Monoclonal antibodies were administered intravenously to 17-day-old BALB/cAnNcR mice at a dose of 250 μg in 0.25 ml per mouse 24 h prior to aerosol challenge. Monoclonal antibody BL-2 was also passively administered to B10.D2/O2Sn and B10.D2/N2Sn mice, obtained from the Jackson Laboratory, Bar Harbor, Maine. Mice were infected with an aerosol of *B. pertussis* 18323 as previously described (17). In some experiments, groups of mice were sacrificed at different times after challenge and dilutions of homogenized lungs and tracheas were plated for bacterial recovery (16). Following infection, the mice were bled, the number of leukocytes per microliter of blood was determined, and any deaths were noted (17). The arithmetic mean and standard deviation of the leukocyte count on the peak day of leukocytosis are reported, and significance between groups receiving test and negative control antibodies was analyzed by Student's *t* test. Statistical significance of survival between groups receiving test and negative control antibodies was determined by chi-square analysis of contingency tables.

The bactericidal activity of the preparations of purified monoclonal anti-LOS A antibodies used in our in vivo experiments is shown in Fig. 1. Monoclonal antibodies BL-1, BL-2, and BL-5 all exhibited greater than 50% reduction of *B. pertussis* CFU, whereas BL-6 and BL-7 did not demonstrate any comparable bactericidal activity despite high ELISA titers, consistent with previous analyses of ascites fluids (1). None of the purified monoclonal anti-LOS A antibodies tested were bactericidal in the presence of heat-inactivated complement.

The ability of these purified monoclonal anti-LOS A antibodies to passively protect against *B. pertussis* aerosol infection was then evaluated. Mice receiving BL-2 (a bactericidal IgG3 anti-LOS A monoclonal antibody) prior to infection had a twofold increase in leukocyte count, compared with uninfected controls, and did not die (Table 1). However, mice receiving

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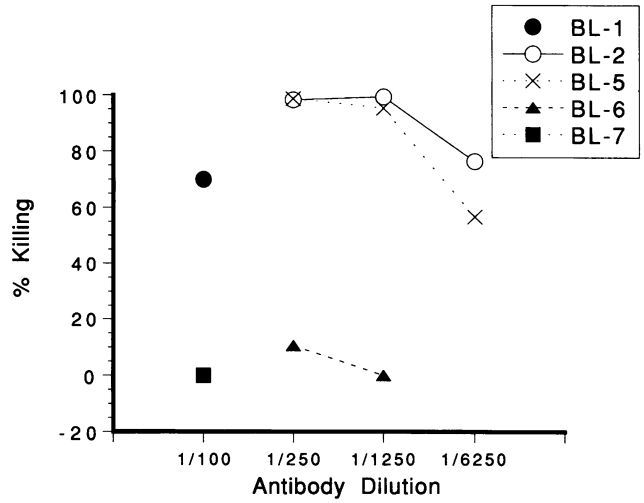

FIG. 1. In vitro bactericidal activity of purified monoclonal anti-LOS A antibodies. BL-2 and BL-5 were tested at starting concentrations of 2.5 mg/ml and had ELISA titers of 50,000 and 4,000, respectively. BL-6 was tested at a starting concentration of 1.3 mg/ml and had an ELISA titer of 200,000. BL-1 and BL-7 were tested at a starting concentration of 1.0 mg/ml and had ELISA titers of 1,000,000 and 10,000, respectively. The in vitro bactericidal activity of these monoclonal antibodies was evaluated with guinea pig serum as a source of complement.

P2-3, as an isotype-matched negative control, exhibited a statistically significant increase in leukocyte count compared with mice receiving BL-2, and 9/10 of the P2-3 group died by day 22. BL-5 is a second IgG3 anti-LOS A monoclonal antibody that, like BL-2, is bactericidal in vitro. Mice receiving BL-5 prior to infection exhibited an increase in leukocyte count with only 12.5% surviving, similar to the low survivor rate and leukocytosis observed in controls receiving P2-3 (Table 1).

Three monoclonal IgG1 anti-LOS A antibodies were also analyzed for their ability to passively protect mice infected with *B. pertussis*. All three of these IgG1 anti-LOS A monoclonal antibodies mitigated leukocytosis in comparison with isotype-matched negative controls (Table 2). BL-1, which exhibited in vitro bactericidal activity (Fig. 1), also protected 100% of infected mice (Table 2). Two additional IgG1 anti-LOS A monoclonal antibodies, BL-6 and BL-7, which did not demonstrate in vitro bactericidal activity, passively protected 66 and 83% of infected mice, respectively.

TABLE 1. Ability of IgG3 anti-LOS A antibodies to protect against *B. pertussis* respiratory challenge

Group	*n*	WBC/μl (10^3)[a]	% Survivors[b]
BL-2	10	45 ± 23 ($P < 0.001$)	100 ($P < 0.001$)
P2-3	10	118 ± 42	10
Uninfected controls	10	21 ± 12	100
BL-5	8	111 ± 52 ($P > 0.05$)	12.5 ($P = 1.000$)
P2-3	9	78 ± 24[c]	11
Uninfected controls	10	17 ± 4	100

[a] At 15 days (BL-2, P2-3) or 16 days (BL-5, P2-3) postinfection. WBC, leukocytes.
[b] At 22 days (BL-2, P2-3) or 21 days (BL-5, P2-3) postinfection.
[c] Leukocyte count for two mice that had survived to day 16. Peak leukocyte count of this group was 92 ± 15 for eight of nine mice alive on day 14.

TABLE 2. Protection by IgG1 anti-LOS A antibodies

Group	*n*	WBC/μl (10^3)[a]	% Survivors[b]
BL-1	12	17 ± 4 ($P < 0.001$)	100 ($P < 0.001$)
BL-7	12	26 ± 8 ($P < 0.005$)	83 ($P < 0.005$)
11B-9	12	90 ± 56	17
Uninfected control	6	6 ± 1	100
BL-6	9	56 ± 25 ($P < 0.001$)	66 ($P < 0.01$)
P2-12	9	152 ± 25	0
Uninfected control	10	17 ± 4	100

[a] At 18 days (BL-1, BL-7, 11B-9) or 16 days (BL-6, P2-12) postinfection. WBC, leukocytes.
[b] At 25 days (BL-1, BL-7, 11B-9) or 21 days (BL-6, P2-12) postinfection.

As BL-2 is bactericidal in the presence of complement in vitro, we tested the requirement for the classical pathway of complement for BL-2-mediated protection in vivo. B10.D2/O2Sn mice are C5 deficient, while B10.D2/N2Sn are C5 competent (19). These mice are smaller than age-matched BALB/c mice, so it was not possible to intravenously inject 17-day-old B10.D2/N2Sn and B10.D2/O2Sn mice. Mice older than 18 to 19 days of age maintained under specific-pathogen-free conditions become infected with *B. pertussis* following aerosol challenge and exhibit a mild leukocytosis, but, in contrast to younger animals, do not die (18). However, Fig. 2 indicates that statistically significant decreases in bacterial recoveries are observed in BALB/c mice that receive BL-2, the same monoclonal antibody that protects against leukocytosis and death (Table 1). A 0.5- to 1.0-$\log_{10}$ CFU decrease in bacterial recoveries from the lungs and tracheas of mice that had received BL-2 compared with mice that had received P2-3 was observed that was statistically significant at all time points analyzed ($P < 0.05$ by Student's *t* test), except for the bacterial recoveries from tracheas at 3 days postinfection (Fig. 2). Therefore, decreases in bacterial recoveries were analyzed from B10.D2/N2Sn (C5-competent) and B10.D2/O2Sn (C5-deficient) mice administered 250 μg of BL-2 intravenously at 20 to 24 days of age and aerosol challenged with *B. pertussis* 24 h later. A group of each strain was sacrificed 7 days after aerosol challenge, and their lungs and tracheas were plated for bacterial recoveries (Fig. 3). B10.D2/O2Sn (C5-deficient) mice exhibited a statistically significant increase in bacterial recovery from the lungs in comparison with B10.D2/N2Sn (C5-competent) mice ($P < 0.005$, two-tailed Fisher's exact test). However, the bacterial recoveries observed in the tracheas of these two mouse strains were not statistically different.

Of the protective anti-LOS A monoclonal antibodies, BL-1 and BL-2 were both found to be bactericidal in vitro in the presence of guinea pig complement, while BL-6 and BL-7, both IgG1 antibodies, had no in vitro bactericidal activity. In contrast, BL-5 was bactericidal in vitro, but not protective in vivo. Thus, the in vitro bactericidal activity of anti-LOS antibodies does not strictly correlate with the ability of these same monoclonal antibodies to mitigate leukocytosis and protect against death following in vivo *B. pertussis* respiratory infection.

On the other hand, the observation that the bactericidal BL-5 did not protect suggests that in vitro bactericidal activity is not a sufficient predictor of in vivo protection. All of the

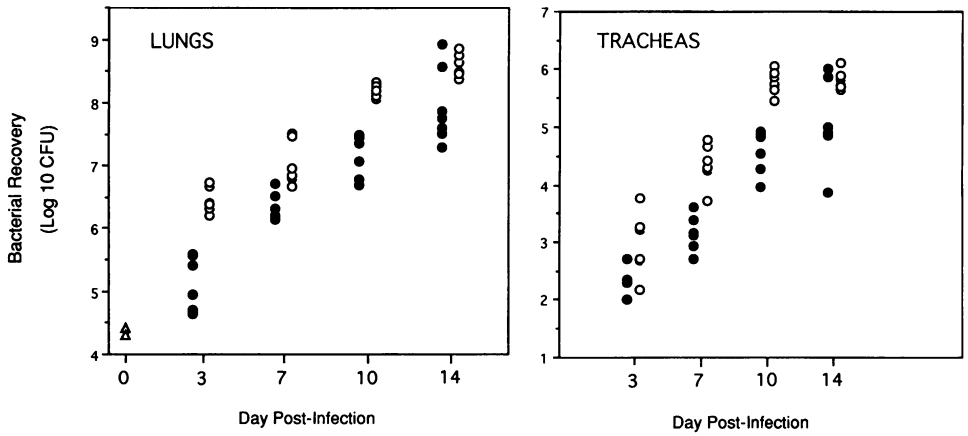

FIG. 2. Bacterial recoveries from the lungs and tracheas of BALB/c mice administered monoclonal antibodies to LOS A prior to *B. pertussis* infection. Each circle represents the bacterial recovery from the lungs or trachea of a single mouse at different times after challenge with an aerosol of *B. pertussis* 18323. Twenty-four hours prior to respiratory infection, mice were administered 250 μg of BL-2 (●) or P2-3 (○). Two uninoculated mice were sacrificed immediately after infection to determine the number of bacteria in their lungs (△).

monoclonal antibodies to LOS A tested have been shown in radiolabelling experiments to bind to the surface of *B. pertussis* (1). Although BL-5 binds to whole *B. pertussis* with sufficient avidity to be bactericidal in vitro, it has approximately 10-fold-less bactericidal and ELISA activity per mg of protein than BL-2 (1). Therefore, the lack of in vivo protection observed with BL-5 may be due to less efficient binding of this monoclonal antibody to *B. pertussis*, which may reflect a decreased affinity of this antibody for its binding site on the bacteria in comparison with protective bactericidal anti-LOS monoclonal antibodies.

The fact that both bactericidal and nonbactericidal monoclonal antibodies were protective suggests that there is more than one operative mechanism of protection mediated by LOS-specific antibodies. In addition to bactericidal activity and neutrophil recruitment mediated by complement-fixing antibodies, such as BL-2 and BL-1, nonbactericidal monoclonal antibodies such as BL-6 and BL-7 may protect by functioning as opsonins that enhance phagocytic activity (6, 9).

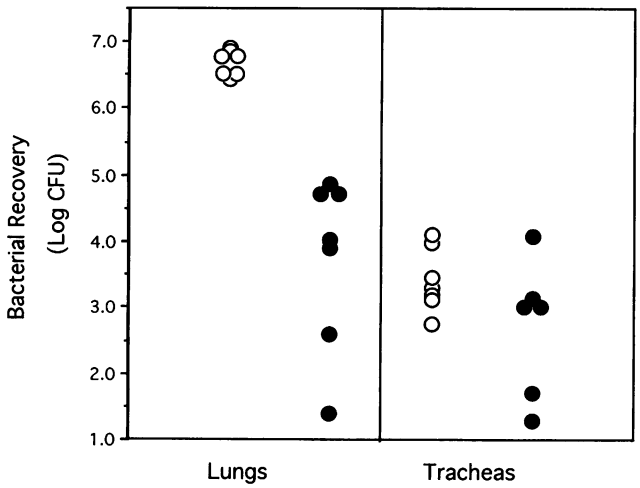

FIG. 3. Bacterial recoveries from the lungs and tracheas of B10.D2/O2Sn (○) or B10.D2/N2Sn (●) mice administered monoclonal antibody BL-2 prior to *B. pertussis* infection. Each circle represents the bacterial recovery from the lungs or trachea of a single mouse 7 days after challenge with an aerosol of *B. pertussis* 18323.

Idiotypic analysis indicated that monoclonal antibodies BL-2, BL-1, BL-6, and BL-7 shared common idiotopes, whereas BL-5 was distinct from the others (3). The lack of protection with BL-5 might suggest a role for idiotypy in antibody-mediated protection against *B. pertussis*; this, however, remains to be defined.

The ability of BL-2 to decrease bacterial infection in the lung was diminished in C5-deficient mice, suggesting that BL-2-enhanced clearance of *B. pertussis* from the lungs involves the fifth component of complement. Activation of the complement cascade by BL-2 may be directly bactericidal, but could also reduce infection by the recruitment of granulocytes to the site of infection through the release of chemotactic fragments of C5 (21).

While IgA has been described as a protective antibody active at mucosal surfaces, such as the upper respiratory tract, specific IgG antibodies have been shown to protect against pneumonia in the lower respiratory tract in several systems (15, 20). *B. pertussis* initially colonizes the ciliated epithelium of the respiratory mucosa, but can proceed to pneumonia, especially in the very young in whom pertussis morbidity and mortality are the greatest. In addition, *B. pertussis* has been found in intracellular association with alveolar macrophages (4, 7); although the significance of this association in the pathogenic process is not yet understood, it has been postulated that it may constitute a carrier state for the disease. Therefore, IgG antibodies specific for surface determinants of *B. pertussis*, such as the protective IgG anti-LOS A antibodies described here, may play an important role in protection of the lower respiratory tract against pertussis pneumonia, as well as perhaps interfering with the natural transmission of disease. Further understanding of the mechanisms of protective immunity to *B. pertussis* infection and the role they play at different stages of pathogenesis will aid in the rational design of future pertussis vaccine candidates.

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