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Role of Antibody to Leukocytosis-Promoting Factor Hemagglutinin and to Filamentous Hemagglutinin in Immunity to Pertussis

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Gamma globulins were obtained from rabbits hyperimmunized with the filamentous hemagglutinin (FHA) or the leukocytosis-promoting factor hemagglutinin (LPF·HA) of *Bordetella pertussis*. Double immunodiffusion analyses showed the antibodies to be highly specific for the homologous antigen. Intraperitoneal injection of anti-FHA or anti-LPF·HA into 10-day-old ICR mice 30 min before aerosol challenge with *B. pertussis* protected the mice from disease as evidenced by survival and a normal rate of gain in body weight. Both anti-LPF·HA and anti-FHA enhanced the clearance of *B. pertussis* from the lung and prevented leukocytosis which normally occurs after aerosol infection of mice with *B. pertussis*. Anti-LPF·HA, but not anti-FHA, neutralized the leukocytosis-promoting and histamine-sensitizing activities of LPF·HA when incubated with the purified protein in vitro. Anti-FHA, but not anti-LPF·HA, prevented the attachment of *B. pertussis* to mammalian cells (HeLa and Vero) in culture.

Since Sauer (26) and Kendrick and Eldering (9) established that effective vaccines containing whole cells of *Bordetella pertussis* could be made, there have been many attempts to identify the protective antigen(s) of *B. pertussis*. After Keogh et al. (11) discovered that cultures of *B. pertussis* produced a hemagglutinin (HA) some studies (5, 10) suggested the HA played an important role in immunity to pertussis, whereas other data (13, 20, 29) suggested that HA was not involved in immunity to pertussis. Recently Sato et al. (23) reported that immunization with a purified preparation containing HA and leukocytosis-promoting factor (LPF) protected mice from intracerebral infection. Arai and Sato (3) subsequently separated this preparation into two distinct HAs.

Once HA (leukocytosis-promoting factor hemagglutinin or LPF·HA) had low hemagglutinating activity and produced leukocytosis and an increased sensitivity to the lethal effects of histamine when injected into mice. This protein showed a single band on polyacrylamide gel electrophoresis and had a molecular weight of about 105,000. Electron micrographs of LPF·HA showed spherical molecules about 6 nm in diameter. Similar preparations of LPF·HA have been purified by Morse and Morse (15) and Irons and MacLennan (7, 8). LPF·HA, when injected into mice, also stimulates the secretion of insulin upon glucose or epinephrine challenge (Y. Sato, J. L. Cowell, H. Sato, and C. R. Manclark, unpublished data) and appears identical to the islet-activating protein of *B. pertussis* (32).

The other HA, filamentous HA (FHA), had high hemagglutinating activity, was free of leukocytosis-promoting and histamine-sensitizing activities, and was antigenically distinct from the LPF·HA (3). Electron micrographs of FHA showed filamentous structures about 2 × 40 nm in size. Gel electrophoresis in sodium dodecyl sulfate showed one major band (molecular weight, 126,000) and several other bands with molecular weights between 120,000 and 52,000. Similar preparations of FHA have been obtained by Morse and Morse (15) and Irons and MacLennan (7). As currently purified, FHA is a protein preparation of multiple components, free of LPF·HA, and enriched in an HA distinct from the LPF·HA. Sato et al. (24) have proposed that the filamentous structures in the FHA are derived from fimbriae which they suggest are HA and adherence factors for *B. pertussis*.

Mouse protective activity was re-examined with these two distinct HAs. Sato et al. (24), Morse and Morse (15) and Irons and MacLennan (7) reported that active immunization with FHA protected mice from intracerebral challenge with *B. pertussis*. LPF·HA made a toxoid by Formalin was a poor protective antigen for mice when tested by Sato et al. (24), and

active LPF·HA was not protective at the doses tested by Irons and MacLennan (7). However, Munoz and Bergman (16, 17) found that a preparation of LPF·HA, referred to as pertussigen and reported to be free of demonstrable FHA (2), protected mice from intracerebral *B. pertussis* infection.

In this paper we present passive immunization data to further evaluate the mouse protective activity of FHA and LPF·HA. Using an aerosol-induced respiratory infection of mice as a laboratory model for pertussis (25), we show that intraperitoneal injection of antibody to FHA or to LPF·HA protects mice from disease. Data relevant to possible mechanisms by which anti-FHA and anti-LPF·HA protect are also presented.

MATERIALS AND METHODS

Bacterial strains and growth. *B. pertussis* strain Tohama, phase I or phase III, was grown at 35°C on Bordet-Gengou agar containing 20% defibrinated bovine blood. The cells from freeze-dried cultures were transferred a maximum of three times before use. On the final passage, cells were allowed to grow for 20 h.

Viable counts were determined by diluting samples in sterile 0.01 M sodium phosphate-buffered saline (pH 7.2) (PBS) and spreading 0.1 ml of the appropriate dilutions on Bordet-Gengou agar plates. Plates were incubated at 35°C for 3 to 4 days. Viable counts were expressed in colony-forming units.

Aerosol infection of mice. Aerosol infection of mice was done as previously described (25). Specific-pathogen-free 10-day-old ICR mice were exposed (without mother) for 30 min to an aerosol generated from 2×10^9 cells of *B. pertussis* strain Tohama, phase I per ml suspended in PBS containing 1% Casamino Acids (Difco Laboratories, Detroit, Mich.). Animals were exposed to the aerosol by spraying 0.4 ml of the bacterial suspension per min at a nebulizer pressure of 1.5 kg/cm^2 with filter sterilized air. A specially built nebulizer was used, but similar aerosols of *B. pertussis* can be obtained by using the standard nebulizer distributed by Fisons Corporation, Bedford, Mass. An outlet tube at the bottom of the chamber removed air to a sterilizing system at a negative pressure of about 1.5 kg/cm^2.

About 20 min after stopping the aerosol generation, the mice were removed from the chamber and placed with mothers in cages with air filter covers. This represented zero time after infection. At this time and appropriate times thereafter, the following procedures were performed. (i) Mice were weighed individually. (ii) Blood was removed from the tail vein of each mouse for estimation of the leukocyte (WBC) count per cubic millimeter of blood (23). (iii) Mice were anesthetized with ether and exsanguinated by heart puncture, and the lungs were removed aseptically. (iv) Lungs were homogenized in sterile PBS and colony-forming units of *B. pertussis* in the lungs were determined by plate count on Bordet-Gengou agar.

Preparation of gamma globulins. New Zealand White rabbits were bled before immunization as a source of normal γ globulins. For the preparation of anti-FHA, the FHA preparation in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) containing 1 M NaCl was emulsified with an equal volume of incomplete Freund adjuvant (Difco Laboratories), and 100 μg of protein was injected into the footpads of each rabbit. After 6 weeks, a booster injection of 100 μg of soluble protein in the above tris(hydroxymethyl)aminomethane-sodium chloride buffer (pH 8.0) was injected subcutaneously, and the animals were bled 2 weeks later. Anti-LPF·HA was prepared as above except that the protein was treated with Formalin (23). Antibody to the crude antigenic mixture of *B. pertussis* was prepared as described for anti-FHA except that each rabbit received 500 μg of protein in the primary injection and 1 mg of protein injected into the ear vein in the secondary injection.

The γ globulin fractions of the normal serum and the antisera were prepared by the methods of Harboe and Ingild (6) except that the diethylaminoethyl-Sephadex chromatography step was omitted. The γ globulin preparations were stored at −20°C at protein concentrations of 38, 34, and 26 mg/ml for anti-LPF·HA, anti-FHA, and normal globulins, respectively. The agglutination titers of anti-LPF·HA and anti-FHA for *B. pertussis* strain Tohama, phase I, tested as described by Sato et al. (23), were 1:40 and 1:2560, respectively.

Effect of passive immunization with anti-FHA or anti-LPF·HA. Anti-FHA, anti-LPF·HA, or normal γ globulin were diluted 1:10 with PBS and 0.2 ml was injected intraperitoneally into mice 30 min before aerosol challenge with *B. pertussis*. At zero time after infection and at appropriate times thereafter the weight of the mice, their peripheral WBC counts, and the viable *B. pertussis* cells in the lungs were determined as stated above.

Attachment of *B. pertussis* to mammalian cells. HeLa and Vero cell cultures were grown on glass cover slips (10.5 by 22 mm) placed in sterile plastic dishes (35 by 10 mm; Falcon Plastics, Oxnard, California) containing 3 ml of the HeLa or Vero cell suspension (about 10^5 cells per ml) in Eagle minimal essential medium containing 10% heat-inactivated fetal bovine serum and 100 U of penicillin per ml. The cell cultures were incubated at 36°C in 5% CO_2 in air for 24 h and then washed with Eagle minimal essential medium lacking penicillin before the addition of *B. pertussis*. Suspensions of *B. pertussis* strain Tohama, phase I or phase III, at 1×10^9 cells per ml in Eagle minimal essential medium were incubated at 23°C for 15 min with or without a 1:40 dilution of anti-FHA, anti-LPF·HA, or normal γ globulin. Portions (2 ml) of these suspensions were then added to the 24-h monolayers of HeLa or Vero cells and incubated for 2 h at 36°C in 5% CO_2 in air. The monolayer cells were then washed several times with Eagle minimal essential medium and either fixed with 3 ml of 95% ethanol and stained with 1% crystal violet or fixed and stained by a deoxyribonucleic acid-staining fluorochrome procedure (4).

Purification of FHA and LPF·HA. The proteins were purified and assayed as described by Arai and Sato (3). The preparations used for these studies were obtained from the second agarose gel filtration step.

Neutralization of the leukocytosis-promoting

and histamine-sensitizing activities of LPF·HA. Purified LPF·HA (10 μg/ml) was mixed with an equal volume of PBS or various dilutions of normal γ globulin, anti-FHA or anti-LPF·HA. After 30 min at 23°C, 0.2 ml portions of the mixtures were injected into the tail veins of 14- to 16-g DDY mice. The WBC count per cubic millimeter of blood was assayed 3 days after injection. On day 4, histamine sensitivity was determined by the intraperitoneal injection of 1 mg of histamine base per mouse. Mortality was used as a measure of histamine-sensitizing activity.

Crude antigenic preparation. A preparation containing many antigens of *B. pertussis* was prepared by ammonium sulfate precipitation of the culture supernatant from 4-day stationary cultures of *B. pertussis* Tohama, phase I (23). Solid ammonium sulfate was added to 1 liter of culture supernatant to 80% saturation (561 g/liter) and stirred at 4°C for 20 min. After 72 h at 4°C, the precipitate was collected by centrifugation (27,000 × *g* for 15 min, 4°C). The precipitate was extracted with 5 ml of 0.01 M sodium phosphate buffer (pH 8.0), centrifuged as above, and the pellet was extracted with 5 ml of 0.1 M tris(hydroxymethyl)aminomethane (pH 8.0) containing 0.5 M NaCl. The supernatants of these two extractions were pooled to give the crude antigen at 4.7 mg of protein per ml. This crude antigen preparation showed about 25 bands after sodium dodecyl sulfate gel electrophoresis with 7.5% gels as described by Weber et al. (31).

Immunodiffusion. Immunodiffusion was done in 1% agarose buffered with 25 mM tris(hydroxymethyl)aminomethane (pH 8.0) containing 0.5 M NaCl. Diffusion took place in a moist chamber at 23°C for about 24 h.

Protein determination. Protein concentrations were estimated with Folin reagent (12) with bovine plasma albumin (Armour Pharmaceutical Co., Scottsdale, Ariz.) as the standard.

RESULTS

Passive protection of mice by anti-LPF·HA and anti-FHA. To evaluate the protective capability of anti-LPF·HA and anti-FHA, each was injected intraperitoneally into mice 30 min before the mice were challenged with *B. pertussis* by aerosol inhalation. Mice receiving anti-LPF·HA or anti-FHA had a rate of gain in body weight equivalent to mice not exposed to *B. pertussis* aerosols (Fig. 1). No deaths occurred in the passively immunized or uninfected groups of mice. In contrast, mice that were injected intraperitoneally with normal γ globulin 30 min before aerosol challenge had a significantly reduced rate of body weight gain (Fig. 1), and 80% of the animals died with 14 days of aerosol inhalation.

Specificity of anti-LPF·HA and anti-FHA. Since anti-LPF·HA and anti-FHA passively protected mice from disease, it was important to evaluate the specificity of these antibody preparations. This was done, in part, by the immunodiffusion of anti-LPF·HA and anti-FHA against a mixture of *B. pertussis* antigens (crude antigen) as shown in Fig. 2. Anti-FHA gave two precipitin bands, both of which gave lines of nonidentity with the precipitin formed by immunodiffusion of anti-LPF·HA against the crude antigen. Two additional extremely faint bands could be seen when more concentrated anti-LPF·HA was used. Anti-LPF·HA and anti-FHA formed single precipitin lines with their homologous antigens, and anti-LPF·HA did not precipitate purified FHA nor did anti-FHA form a precipitin line with purified LPF·HA (data not shown). Based on precipitin analyses, anti-LPF·HA and anti-FHA are distinct from each other. In addition, anti-LPF·HA appears specific for LPF·HA, whereas anti-FHA appears specific for two different antigens in the FHA preparation.

Studies concerning the mechanism(s) of protection. Aerosol infection of mice with *B. pertussis* results in an increase in the peripheral WBC count and in the number of *B. pertussis*

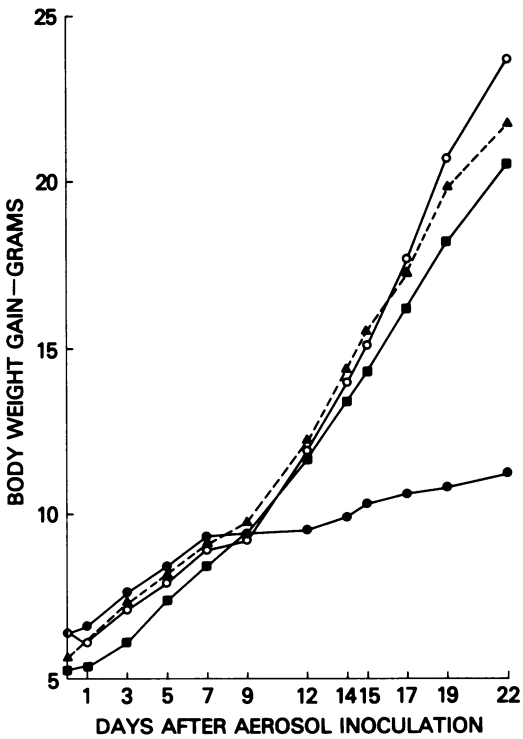

FIG. 1. *Effect of anti-LPF·HA and anti-FHA on the rate of gain in body weight by mice after aerosol inhalation of B. pertussis. Data points represent the mean value from 10 mice, weighed individually. When death occurred, the body weight of the mouse at death was included in calculating the mean body weight per group. Symbols: ▲, noninfected; ○, anti-LPF; ■, anti-FHA; ●, normal γ globulin.*

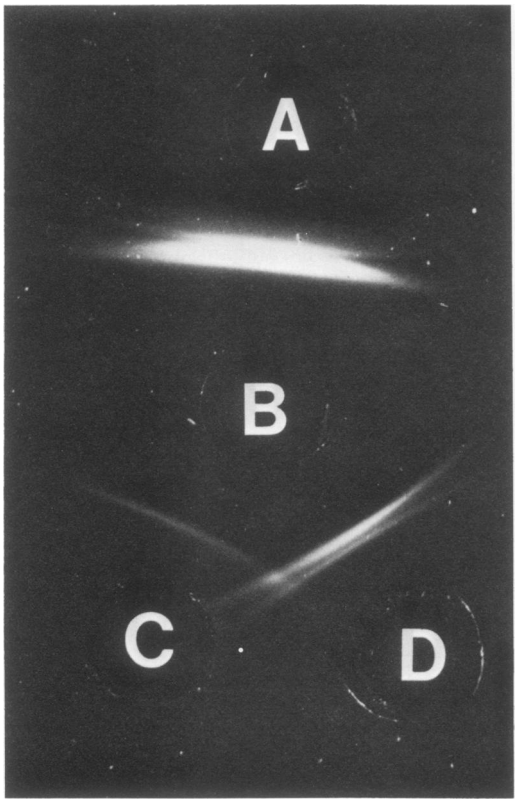

FIG. 2. *Double immunodiffusion. Well A, 5 μl of a 1:5 dilution of anti-crude antigen; well B, 5 μl of crude antigen (4.7 mg/ml); well C, 5 μl of a 1:2 dilution of anti-LPF·HA; well D, 5 μl of anti-FHA.*

cells in lungs (25). Thus, studies were done to determine if passive immunization with anti-LPF·HA or anti-FHA would prevent or attenuate these increases (Fig. 3). As expected from previous data (25), mice injected with normal γ globulin showed an increase in both the WBC count and *B. pertussis* cells in the lung. All of these mice were dead within 18 days after challenge. In mice receiving anti-LPF·HA or anti-FHA, there were no deaths or other signs of illness, and the number of viable cells of *B. pertussis* per mouse lung decreased from 2×10^4 to about 1×10^2 colony-forming units 7 days after inhalation. However, *B. pertussis* was not completely cleared from the lung at up to 27 days after infection. There was no leukocytosis in mice injected with anti-LPF·HA, and only a twofold increase in the WBC count in mice receiving anti-FHA.

When injected into animals, LPF·HA causes leukocytosis and lymphocytosis (3, 8, 12) and is the likely cause of leukocytosis observed after aerosol infection. Since both anti-LPF·HA and anti-FHA prevented leukocytosis in aerosol-infected mice, it was appropriate to determine if anti-LPF·HA and anti-FHA could neutralize the leukocytosis-promoting and histamine-sensitizing activities of the LPF·HA. The neutralization data are shown in Table 1. At a final dilution of 1:36 for anti-LPF·HA, neutralization of LPF·HA activity was complete and significant neutralization was obtained at 1:108. Normal γ globulin or anti-FHA at dilutions at 1:4 did not affect the activities of LPF·HA.

It has been proposed by Sato et al. (24) that FHA contains an adherence factor derived from the fimbriae of *B. pertussis*. This proposal and the observation that anti-FHA and anti-LPF·HA prevented an increase of *B. pertussis* cells in the lungs of aerosol-infected mice prompted the study of the effect of anti-FHA and anti-LPF·HA on the attachment of *B. pertussis* to monolayer cultures of HeLa and Vero cells. Incubation of monolayers with *B. pertussis* strain Tohama, phase I, resulted in attachment of many bacterial cells to the mammalian cells (Fig. 4A), whereas nonvirulent *B. pertussis* strain Tohama, phase III, showed little attachment (Fig. 4B).

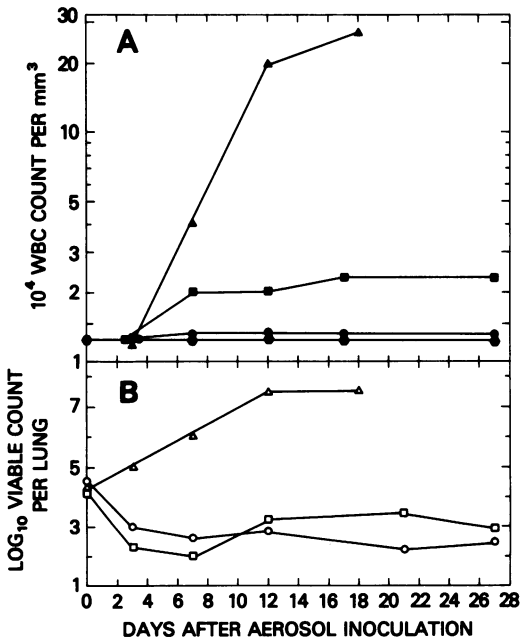

FIG. 3. *Effect of anti-LPF·HA and anti-FHA on the WBC count and viable B. pertussis cell count in the lungs of mice after aerosol infection. Each point showing B. pertussis colony-forming units per lung is a geometric mean from four mice. Each point showing WBC counts is an arithmetic mean from 10 mice. Symbols in (A): ▲, normal γ globulin; ●, anti-LPF·HA; ■, anti-FHA; ⬢, noninfected. Symbols in (B): △, normal γ globulin; ○, anti-LPH·HA; □, anti-FHA.*

TABLE 1. *Effect of anti-LPF·HA and anti-FHA on the leukocytosis-promoting and histamine-sensitizing activities of LPF*[a]

Dilutions	Normal γ globulin		Anti-FHA		Anti-LPF	
	WBC	HS	WBC	HS	WBC	HS
1:4	78×10^3	5/5	84×10^3	5/5	27×10^3	0/5
1:12	71×10^3	5/5	97×10^3	5/5	28×10^3	0/5
1:36	86×10^3	5/5	92×10^3	5/5	27×10^3	0/5
1:108			99×10^3	5/5	40×10^3	1/5
1:324			104×10^3	5/5	58×10^3	3/5
1:972					73×10^3	4/5
1:2916					98×10^3	5/5

[a] Final dilutions of the γ globulin preparations after the addition of an equal volume of LPF·HA (10 μg) are described in the text. WBC (White blood cell count) values are expressed per cubic millimeter of blood and represent arithmetic means from five mice; mice injected with PBS averaged 22×10^3 WBC per mm^3, and mice injected with LPF mixed with PBS gave 78×10^3 WBC per mm^3. HS (histamine sensitivity) values show the number of deaths per total number of animals injected; mice injected with PBS had an HS of 0/5, whereas mice injected with LPF·HA mixed with PBS had an HS of 5/5.

Preincubation of *B. pertussis* strain Tohama phase I cells with anti-FHA appeared to prevent attachment of these cells to the monolayer (Fig. 5A), but preincubation of Tohama phase I cells with anti-LPF·HA did not prevent attachment (Fig. 5B). Photomicrographic fields of HeLa or Vero cells incubated with *B. pertussis* strain Tohama phase I cells which were preincubated with normal γ globulin were similar to Fig. 5B in that many bacteria were attached to the mammalian cells. Figures 4 and 5 show representative photomicrographic fields of results obtained with HeLa cells stained with crystal violet. Similar results were obtained with Vero cells.

DISCUSSION

The data presented show that mice infected by aerosol inhalation of *B. pertussis* were passively protected from disease by two distinct antibody preparations, anti-LPF·HA and anti-FHA. Although more information is required on the mechanism(s) by which anti-LPF·HA and anti-FHA protect, there are several possible explanations. We found that anti-LPF·HA neutralized the leukocytosis-promoting and histamine-sensitizing activities of purified LPF·HA, and it is possible that anti-LPF·HA protects from disease by neutralizing the potentially harmful activities of LPF·HA released during the infectious process. LPF·HA is released from the bacterial cell during growth in vitro (23). Also, since anti-LPF·HA and anti-FHA enhanced the clearance of *B. pertussis* from mouse lungs, it is possible that either or both act to increase phagocytosis of *B. pertussis* by pulmonary phagocytes. In vitro data show that alveolar macrophages (18) and polymorphonuclear leukocytes (30) are activated by antisera to *B. pertussis* to phagocytize virulent *B. pertussis* organisms. In addition, anti-FHA may protect by preventing attachment of *B. pertussis* to respiratory epithelial cells. We did find that anti-FHA prevented the attachment of *B. pertussis* to mammalian cells in vitro, and there are data suggesting that attachment is a virulence factor for *B. pertussis* (14, 24). Matsuyama (14), in studying excised rabbit trachea, showed that cells of virulent *B. pertussis* strain Tohama, phase I, were not moved by ciliary activity from the point of inoculation, whereas nonvirulent *B. pertussis* Tohama phase III cells inoculated at the same area of the tracheal segment were cleared toward the laryngeal end of the trachea.

The fact that anti-FHA and anti-LPF·HA passively protected mice from disease after respiratory infection corroborates active immunization data identifying FHA as a protective antigenic preparation (7, 24), and suggests that immunization with LPF·HA should protect mice from infection with *B. pertussis*. Purified LPF·HA is lethal at relatively low concentrations, and Irons and MacLennan (7, 8), by using the standard mouse potency assay, found the lethal dose of LPF·HA to be smaller than the protective dose. Sato et al. (24) made LPF·HA a toxoid with Formalin, but the toxoid poorly protected mice from intracerebral challenge with *B. pertussis*; it is possible that this toxoid was a poor immunogen. The LPF preparation called pertussigen by Munoz and Bergman (16, 17) has been reported to actively protect mice from intracerebral challenge with *B. pertussis*; however, the purity of this preparation has not been adequately described, and it is not certain whether the LPF·HA in this preparation is acting as a protective antigen or is enhancing the effect of other protective antigens in the sample. Nakase and Doi (19), by using the mouse intracerebral challenge potency test, have shown that small amounts of LPF·HA could enhance the protective effect of FHA. Additional active immunization studies with purified, well-characterized, and appropriately inactivated LPF·HA are needed.

In this discussion of protective antigens and antibody preparations, two different challenge routes, intracerebral injection and aerosol inhalation, have been used. Our passive protection data were obtained from an aerosol induced respiratory infection, whereas the active immu-

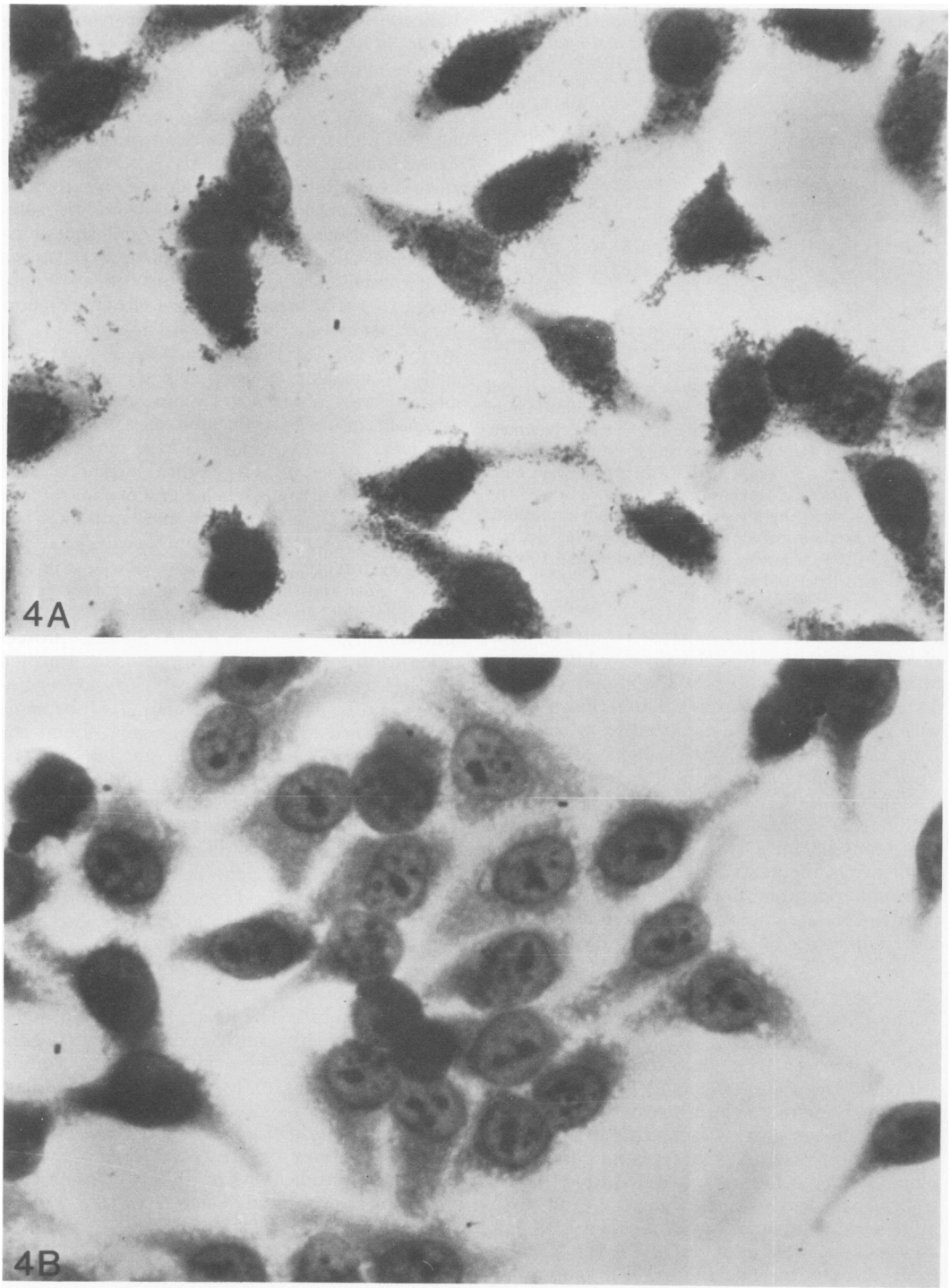

FIG. 4. *Photomicrographs of HeLa cells incubated with B. pertussis strain Tohama phase I (A) or phase III (B) cells. Many B. pertussis cells are visible on the cytoplasmic membranes in photomicrograph A. Cells were crystal violet stained and are shown at a magnification of ×100.*

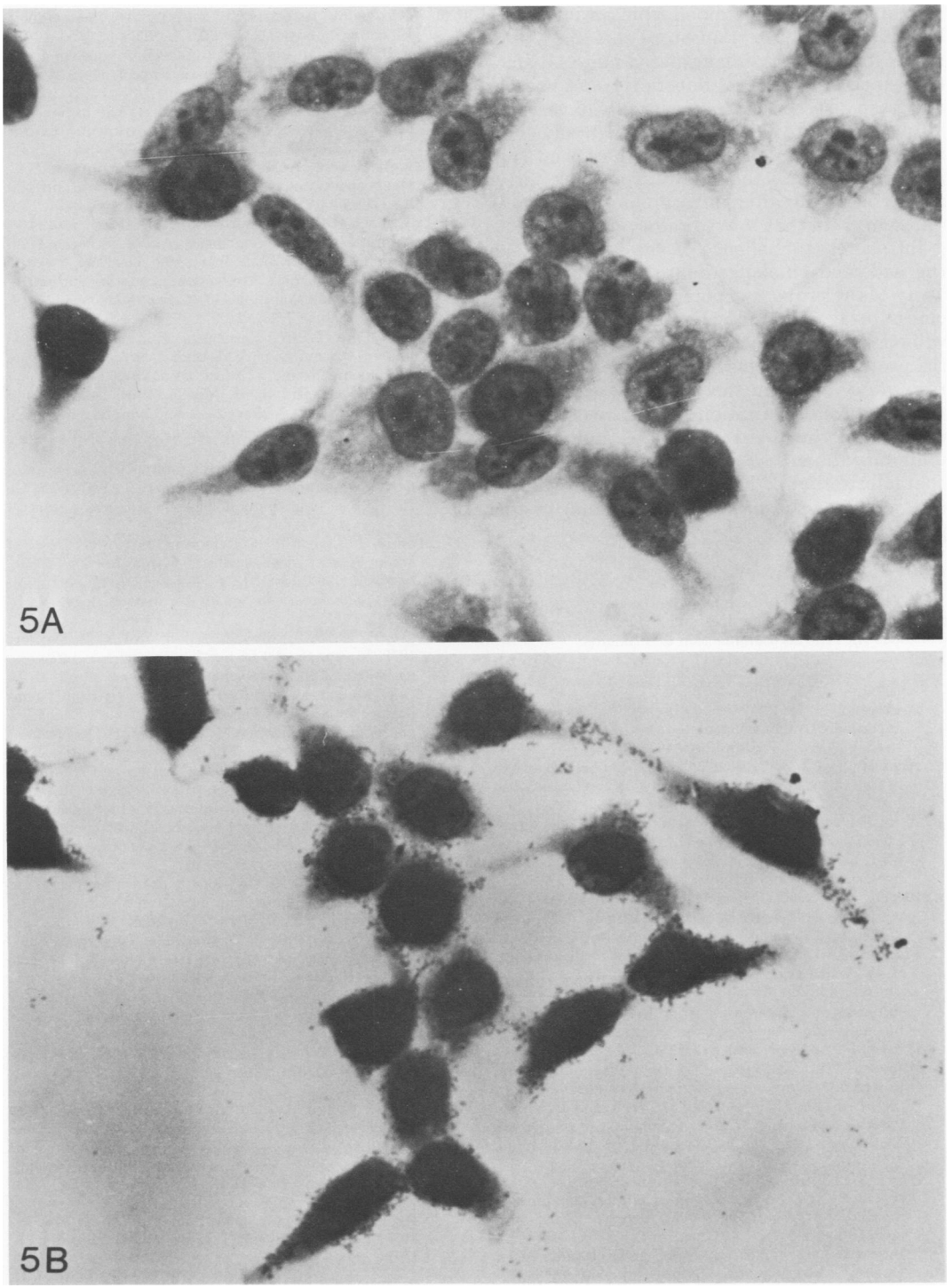

FIG. 5. *Photomicrographs of HeLa cells incubated with B. pertussis strain Tohama phase I cells which were preincubated with anti-FHA (A) or anti-LPF (B). Many B. pertussis cells are visible on the cytoplasmic membranes in photomicrograph B. Cells were crystal violet stained and are shown at a magnification of ×100.*

nization data were obtained from an intracerebral infection. The respiratory infection, as a model for pertussis, is considered more satisfactory since intracerebral infection is not natural and fresh disease isolates are usually of very low virulence when injected intracerebrally into mice (1, 21, 27). In addition, it has been suggested (22, 27, 28) that the host-parasite interaction occurring after intracerebral infection is different from that in respiratory infection.

Intracerebral challenge of vaccinated mice is the accepted challenge route in the standard assay of the protective potency of pertussis vaccines. Intracerebral infection is widely used and will continue to be employed to evaluate potential protective antigens of *B. pertussis*; however, respiratory infection should also be used in further active immunization studies necessary to understand the role of LPF·HA and FHA in immunity to pertussis. In addition, biochemical characterization of FHA is needed to evaluate the nature of the protective antigen(s) in this preparation.

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