INFECTION AND IMMUNITY, May 1983, p. 523-528
0019-9567/83/050523-06$02.00/0

Vol. 40, No. 2

Synergistic Effect of *Bordetella pertussis* Lymphocytosis-Promoting Factor on Protective Activities of Isolated *Bordetella* Antigens in Mice

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Received 28 October 1982/Accepted 9 February 1983

The effect of low levels of added lymphocytosis-promoting factor (LPF) on the ability of several antigenic preparations isolated from *Bordetella pertussis* and other bacteria to protect mice against intracerebral infection with *B. pertussis* was examined. LPF was found to enhance the protective activities of filamentous hemagglutinin, 22S antigen, and fimbriae isolated from *B. pertussis*. Outer membrane protein preparations from phase I *B. pertussis* which had LPF removed by haptoglobin affinity columns or inactivated by glutaraldehyde, sodium dodecyl sulfate, or Formalin had reduced protective activities but were made fully protective by the readdition of LPF. Similarly, outer membrane protein preparations from *Bordetella bronchiseptica, Bordetella parapertussis*, or phase IV *B. pertussis* lacking LPF were protective only when low levels of LPF were added to the preparations. Outer membrane protein preparations from *Neisseria gonorrhoeae* or *Escherichia coli* were nonprotective even in the presence of added LPF. The purified LPF by itself was nonprotective unless treated with glutaraldehyde. LPF that had been detoxified with glutaraldehyde was, however, ineffective at enhancing the protective activity of antigenic preparations. The synergistic effect of LPF is discussed in relation to its known biological properties.

The intracerebral (i.c.) mouse protection test is a mandatory potency assay for pertussis vaccines and has been used to demonstrate the correlation between vaccine protection in mice and children (10). However, the isolation of protective antigens from *Bordetella pertussis*, when monitored by the mouse i.c. potency assay, has led to conflicting results from different laboratories. Recent work has shown that the filamentous hemagglutinin (FHA) is capable of protecting mice against i.c. challenge with *B. pertussis* (6, 22). This work has been questioned by Munoz et al. (14), who maintain that FHA is usually contaminated with very small amounts of a protein known variously as the lymphocytosis-producing factor hemagglutinin (LPF), histamine-sensitizing factor, islet-activating protein, pertussis toxin, or pertussigen. According to Munoz et al., FHA, when purified and free from any demonstrable pertussigen, does not protect mice, whereas preparations rich in pertussigen or purified pertussigen detoxified with glutaraldehyde have high mouse-protective activity. These results, along with data from passive protection tests, led Munoz et al. to the conclusion that pertussigen is the major mouse-protective antigen (14). However, highly purified native pertussigen was found to be nonprotective when tested at doses permitted by its toxicity (6, 13), and furthermore, FHA free from pertussigen has been found to be protective when a double immunization schedule is used (23).

We have recently found that LPF (a term used here synonymously with pertussigen) is important for i.c. protective activity of various outer membrane protein (OMP) preparations of *B. pertussis* (18). When the LPF is removed from OMP preparations by haptoglobin affinity columns, the protective activity of the OMP is reduced dramatically. Readdition of purified LPF restores the protective activity. Similarly, Nakase and Doi (16) found that mixtures of FHA and LPF, neither of which have protective activity alone, give significant protection against i.c. challenge.

In this report, we present data showing that a synergistic effect of LPF on protection, as measured by the i.c. potency assay, is not confined to FHA or phase I *B. pertussis* OMP but occurs with a variety of other *Bordetella* surface anti-

gens. A possible mechanism for this synergistic effect in the mouse i.c. protection test is discussed.

MATERIALS AND METHODS

Bacterial strains and cultural procedures. *B. pertussis* Wellcome 28 was used principally for the preparation of antigens. *Bordetella bronchiseptica* APM 21 and NCTC 8344, *Bordetella parapertussis* NCTC 8250, and *B. pertussis* strains were maintained as freeze-dried cell suspensions and recovered by growth on charcoal agar plates containing 10% (vol/vol) defibrinated horse blood. Cells from plates were inoculated into 100 ml of the medium described by Sato et al. (21) and incubated at 35°C for 24 h on an orbital shaker (180 rpm). Thompson bottles containing 300 ml of medium were inoculated with 10 ml of primary culture and either shaken on a gently reciprocating shaker at 35°C for 16 to 24 h for *B. bronchiseptica* or 36 to 40 h for *B. pertussis* and *B. parapertussis* or incubated statically for 5 days at 35°C. For *B. pertussis* growth in the C mode, the NaCl in the medium was replaced with 5.3 g of $MgSO_4\ 7H_2O$ per liter. After growth, cells were harvested by centrifugation and stored at −20°C.

Escherichia coli HB101 was grown as overnight cultures in L broth.

Neisseria gonorrhoeae P9 was grown for 40 h on plates of gonococcal clear typing medium (24) and harvested by scraping and washing.

Preparation of antigens. (i) OMPs. Envelopes were prepared by Braun homogenization and extracted with the surfactant Empigen BB (Marchon Div., Albright and Wilson, Whitehaven, England) to produce the crude OMP preparations (19, 20) used in the mouse protection test.

The lymphocytosis-promoting activity of OMP preparations was chemically modified by one of the three following methods: (i) glutaraldehyde treatment after the separation of protein and lipopolysaccharide (LPS) components of OMP preparations (18); (ii) sodium dodecyl sulfate treatment in which 12 ml of an OMP preparation was concentrated by pressure dialysis to 4 ml, 0.8 ml of 10% (wt/vol) sodium dodecyl sulfate in 0.05 M sodium phosphate (pH 8.0) was added, and the preparation then incubated at 37°C for 30 min; or (iii) formaldehyde treatment in which 3 ml of OMP (1.75 mg of protein per ml) was mixed with 40 μl of 40% formaldehyde and incubated at 4°C for 3 days.

LPF was removed from purified OMP preparations that were free from LPS by affinity chromatography on haptoglobin coupled to Sepharose 4B (18).

(ii) FHA. FHA was purified from culture supernatants of 5-day static growths of *B. pertussis* (6, 19). It was also purified from 50 mM Tris–1 M NaCl (pH 8.0) buffer extracts of 72-h growths of *B. pertussis* or 48-h growths of *B. bronchiseptica* APM 21 on charcoal agar medium (L. Irons, manuscript in preparation).

(iii) LPF. The affinity chromatography method of Irons and MacLennan (7) was used to purify LPF from Dynomill or Braun cell disintegrates of *B. pertussis*. Purified LPF was treated with glutaraldehyde to destroy its toxicity as described by Munoz et al. (14).

(iv) *B. pertussis* 22S antigen. The 22S antigen of *B. pertussis* was purified from cell disintegrates by differential centrifugation and Sepharose 6B and DEAE-cellulose chromatography (11).

(v) Fimbriae. The release of fimbriae (agglutinogens) from *B. pertussis* Wellcome 28 (serotype 1, 2, 3) by homogenization was monitored by measuring inhibition of bacterial agglutination with a serotype 2-specific antiserum (3). The fimbriae were purified from the homogenate by Sepharose 6B chromatography.

(vi) LPS. LPS was purified from *B. pertussis* by the phenol-water method as described by MacLennan (9).

Analytical procedures. The protein content of antigen preparations was determined by an automated Lowry method.

Mouse protection test. The mouse i.c. challenge test of Kendrick et al. (8) was used as follows. Five milliliters of diluted antigen was mixed with 5 ml of Alhydrogel (Superfos Export Company A/S, Vedbaek, Denmark) and rotated for 1 h at 4°C before centrifuging and dispersing the pellet in phosphate-buffered saline to 15 ml. Alternatively, 5 ml of Alhydrogel was centrifuged and the pellet was dispersed in phosphate-buffered saline to 10 ml and then mixed directly with 5 ml of diluted antigen which had been previously dialyzed against phosphate-buffered saline. When required, purified LPF was added to the antigen before treatment with Alhydrogel. The Alhydrogel suspensions were then serially diluted with phosphate-buffered saline so that the ratio of antigen to LPF-Alhydrogel remained constant for each dilution of a particular vaccine. Mice in groups of 20 were injected intraperitoneally with 0.5 ml of vaccine and challenged i.c. 14 days later with about 5×10^4 organisms (1,000 × 50% lethal dose) of *B. pertussis* 18-323. Deaths were recorded after 14 days, and the 50% protective dose (PD_{50}) value was obtained from the dose-response curve by probit analysis.

LPF assay. The lymphocytosis-promoting activity of antigens was determined in 5-week-old male Porton mice as described by Morse and Morse (12). Protein (50 to 300 μg per mouse) was injected, and the resultant lymphocytosis was compared with that induced by purified LPF injected at doses of 0.01 to 1 μg.

RESULTS

Effect of LPF on the protective activity of phase I *B. pertussis* antigens. Purified LPF at doses of 0.001 to 1 μg per mouse was found to be nonprotective against i.c. challenge with *B. pertussis* (Table 1).

FHA purified from static culture supernatants had a PD_{50} value of 3 μg of protein (Table 1) and was contaminated with about 0.3% LPF. FHA produced from *B. pertussis* cells grown on a solid medium had a higher PD_{50} value but was contaminated with less than 0.1% LPF. Attempts to separate all the contaminating LPF from FHA by affinity columns of haptoglobin coupled to Sepharose 4B or by treatment with Triton X-100 (14) were not successful. Both methods removed only about 50% of the contaminating LPF, and there was little change in mouse-protective activity (data not shown). However, an increase in protective activity

TABLE 1. Effect of added LPF on the protective activities of various antigen preparations from phase I *B. pertussis*

Prepn	LPF activity (%)[a]	PD_{50} (μg of protein per mouse)[b]		Antigen/LPF ratio[c]
		Without LPF	With LPF	
LPF	100	>1*		
FHA (liquid culture)	0.3	3.0	0.8	51/0.5
FHA (solid culture)	0.06	18.9	4.0	96/0.5
OMP	0.2	2.1 ± 1.2 (42)	ND[d]	ND
OMP-haptoglobin treated	<0.01	24.6 ± 2.1 (5)	1.6 ± 1.4 (4)	50/0.2
OMP-glutaraldehyde treated	<0.01	16.7	1.16	50/0.2
OMP-sodium dodecylsulfate treated	<0.05	47.7	2.1	50/0.2
OMP-formaldehyde treated	0.02	11.8	2.1	50/0.2
22S antigen	0.05	>73*	8.1	73/0.5
Fimbriae	ND	>5*	2.7	5/0.5

[a] Approximate indigenous LPF activity of the preparation, estimated by comparing the lymphocytosis induced in mice with that induced by purified LPF.

[b] PD_{50}, Dose that protected 50% of the mice challenged i.c.; *, nonprotective at highest dose tested. Results are shown as mean ± standard error of the mean; number of determinations is shown in parentheses.

[c] Micrograms of antigen divided by micrograms of added LPF injected into each mouse at the highest dose. The indigenous LPF is not taken into account.

[d] ND, Not determined.

could be obtained by the addition of small amounts of purified LPF to FHA isolated from cells grown in either liquid or solid medium (Table 1).

When OMP preparations from *B. pertussis* were treated with haptoglobin affinity columns, it was found that both LPF and protective activities were reduced. Readdition of purified LPF to the OMP restored these activities (Table 1). Similar results were obtained by adding LPF to OMP preparations whose lymphocytosis-promoting activity had been modified by treatment with glutaraldehyde, sodium dodecyl sulfate, or Formalin (Table 1). In one experiment, OMP was treated with various levels of glutaraldehyde, and it was found that the loss of protective activity depended on the glutaraldehyde/OMP ratio. However, when LPF was added, all glutaraldehyde-treated OMP preparations were restored to approximately the same protective activity (data not shown).

A pronounced effect on mouse-protective activity was also found when LPF was mixed with the 22S antigen or fimbriae purified from *B. pertussis* (Table 1). LPS purified by the phenol-water method was nonprotective when tested at various doses of 0.5 to 100 μg per mouse. Injection of 20 or 5 μg of LPS mixed with 0.5 μg of LPF gave a marginal increase in protection (survival rates of 25 to 30%), whereas other mixtures of LPS and LPF showed no protective activity (data not shown).

Effect of LPF on the protective activity of antigens from phase IV *B. pertussis* and other bacteria. The increase in protective activity found when LPF was added to various phase I *B. pertussis* antigens led us to test the effect of LPF on OMP preparations from phase IV *B. pertussis* strains which were naturally devoid of LPF. The OMP preparations from three phase IV *B. pertussis* strains and from *B. pertussis* Wellcome 28 grown in the C mode possessed no detectable protective activity (Table 2) and had negligible LPF activity. However, addition of 0.2 μg of LPF to 50 μg of each type of OMP raised its protective activity to a value equivalent to that of phase I *B. pertussis* OMP (Table 2).

We observed similar effects of LPF on the abilities of OMP preparations from *B. bronchiseptica* and *B. parapertussis* and of FHA from *B. bronchiseptica* to protect against i.c. challenge with *B. pertussis* (Table 3). However, no protection against *B. pertussis* was found with either *E. coli* or *N. gonorrhoeae* OMP in the presence of LPF (Table 3), showing that protective activity appeared to be limited to the *Bordetella* genus.

Glutaraldehyde treatment of LPF. Native LPF was toxic at levels of about 2 μg per mouse, and the dose required to double the leukocyte count of a mouse was approximately 0.02 μg. After treatment with glutaraldehyde, 10 μg of LPF was nontoxic to mice and produced only a marginal increase in leukocyte count. Glutaraldehyde-treated LPF had a PD_{50} value of 2.9 μg of protein. No protective activity was found with native LPF at 1 μg per mouse, which was the highest dose tested, owing to the toxicity of LPF. The detoxified LPF, at 0.5 or 0.2 μg per mouse, did not increase the protective activity of FHA or of OMP preparations treated with haptoglobin affinity columns (Table 4).

Effects of variation of the immunization proce-

TABLE 2. Effect of added LPF on protective activities of OMP preparations from phase IV and C-mode *B. pertussis*

B. pertussis strain for OMP prepn	PD_{50} (μg of protein per mouse)[a]		Antigen/LPF ratio[b]
	Without LPF	With LPF	
134, Phase IV	>50* (2)	0.62 ± 1.6 (2)	50/0.2
Wellcome 28, Phase IV	>50* (3)	1.3 ± 1.1 (3)	50/0.2
10901, Phase IV	>50*	0.55	50/0.2
Wellcome 28, C mode	>50* (2)	0.48 ± 2.4 (2)	50/0.2

[a] PD_{50}, Dose that protected 50% of the mice challenged i.c.; *, nonprotective at highest dose tested. Results are shown as mean ± standard error of the mean; number of determinations is shown in parentheses.

[b] Micrograms of antigen divided by micrograms of added LPF injected into each mouse at the highest dose. The indigenous LPF is not taken into account.

dure. In the protection tests described above, mice were immunized with mixtures of LPF and antigen in Alhydrogel. Experiments aimed at varying the immunization procedure, in which phase IV *B. pertussis* OMP was used as antigen, showed the following. (i) OMP and LPF did not need to be mixed but could be injected into mice at separate sites. (ii) A variable effect of LPF on mouse-protective activity could be obtained by injecting LPF before or after the antigen. For example, if mice were injected with dilutions of antigen 14 days before challenge, the preparation was found to be nonprotective. When 0.05 μg of LPF was injected into all mice on the same day as the antigen, the PD_{50} value was 0.39 μg of protein. When the LPF was injected 3 days before or 7 days after the antigen, respective PD_{50} values of 1.31 and 9.7 μg of protein were obtained. Thus, the maximum effect occurred when LPF was injected on the same day as the antigen. (iii) The magnitude of the effect was dependent on the dose of LPF injected. When groups of mice were injected with a constant amount of antigen (10 μg), the highest protective activity was obtained when at least 0.04 μg of LPF was also injected.

TABLE 3. Effect of added LPF on the activities of various bacterial preparations protecting against i.c. *B. pertussis* challenge

Prepn	PD_{50} (μg of protein per mouse)[a]		Antigen/LPF ratio[b]
	Without LPF	With LPF	
B. bronchiseptica NCTC 8344 OMP	>50*	2.7	50/0.5
B. bronchiseptica APM 21 OMP[c]	>50*	2.1	50/0.5
B. parapertussis NCTC 8250 OMP	>50*	1.1	50/0.2
N. gonorrhoea P9 OMP	>50*	>50*	50/0.2
E. coli HB101 OMP	>50*	>50*	50/0.2
B. bronchiseptica APM 21 FHA	>18*	2.9	18/0.5

[a] PD_{50}, Dose that protected 50% of the mice challenged i.c.; *, nonprotective at highest dose tested.

[b] Micrograms of antigen divided by micrograms of added LPF injected into each mouse at the highest dose. The indigenous LPF is not taken into account.

[c] *B. bronchiseptica* APM 21, a strain producing FHA isolated from vervet monkeys (6).

DISCUSSION

Nakase and Doi (16) have reported that mixtures of FHA and LPF, neither of which have protective activity alone, give strong protection to mice against i.c. challenge with *B. pertussis*. Sato et al. (23) also found that mixtures of FHA and LPF are highly protective. Our results showed that other proteins which have little or no demonstrable mouse i.c. protective activity can be made highly protective by including low levels of LPF in the vaccines. The proteins affected by LPF are purified fimbriae and 22S antigen, OMP preparations of phase I *B. pertussis* which have LPF removed or inactivated, and FHA or OMP preparations from *B. bronchiseptica*, *B. parapertussis*, and phase IV *B. pertussis* which are naturally devoid of LPF.

It has been suggested that the protective activity of FHA (6, 22) is due to contamination of preparations with small amounts of LPF that are difficult to remove and that LPF is the major mouse-protective antigen (14). However, it is difficult to reconcile in a simple manner the protection conferred by FHA or OMP preparations with the measured degree of LPF contamination, which is between 0.1 and 0.5%. This degree of contamination would mean a specific PD_{50} value for LPF in these preparations of about 0.002 to 0.01 μg of protein. LPF is not protective at these or higher levels. Thus, to explain our results, the protective activity of LPF would have to be considerably enhanced by combination with other *B. pertussis* antigens. No enhancement occurred with *N. gonorrhoeae* or *E. coli* antigens. The observation that active LPF can be given before or after the candidate

TABLE 4. Effect of glutaraldehyde treatment on the synergistic effect and protective activity of LPF

Prepn	PD_{50} (μg of protein per mouse)[a]			Antigen/LPF ratio[b]
	Without LPF	With LPF	With Glt LPF	
LPF	>1*	ND	ND	
Glt LPF	2.9 ± 1.6 (3)	ND	ND	
FHA (solid culture)	17.8	1.6	24.6	36/0.5
OMP-haptoglobin treated	>50*	2.2	>50*	50/0.2

[a] PD_{50}, Dose that protected 50% of the mice challenged i.c.; *, nonprotective at highest dose tested; Glt, glutaraldehyde-treated LPF; ND, not determined. Results are shown as mean ± standard error of the mean; number of determinations is shown in parentheses.

[b] Micrograms of antigen divided by micrograms of added LPF (native or glutaraldehyde treated) injected into each mouse at the highest dose.

protective antigens indicates that a physical complex between antigen and LPF is not required for protection.

We suggest that a more plausible explanation is the converse, i.e., that the protective activities of the other antigens are enhanced in the presence of LPF. The mechanism by which LPF produces this synergistic effect is not yet understood. It does not appear to involve the well-known adjuvant effect of LPF on levels of circulating antibody (manuscript in preparation) and most likely arises from the ability of LPF to increase the vascular permeability of the blood-brain barrier (1). The possible importance of this effect in protection against i.c. challenge with *B. pertussis* has been discussed by Munoz and Bergman (15). That the synergistic effect of LPF is related to its pharmacological actions also receives support from the following observations: (i) Glutaraldehyde-treated LPF, which has many of its biological activities reduced or destroyed (13), does not exhibit a synergistic effect with OMP or FHA; and (ii) native LPF produces a synergistic effect when injected 3 days before the antigen or 7 days before challenge.

The fact that LPF enhances the protective activities of antigens from *B. bronchiseptica, B. parapertussis*, and phase IV *B. pertussis* suggests the presence of common antigens in the preparations. In might be considered that LPS is such a common antigen, since it has been shown (5) that i.c. injection of *B. pertussis, B. bronchiseptica*, or *Salmonella typhimurium* LPS into mice confers nonspecific protection against i.c. challenge with *B. pertussis*. This seems unlikely, however, since the nonspecific protection of LPS reaches a maximum 2 days after immunization (5) and is absent when the interval from immunization to challenge is extended to 14 days (4). Also, as shown in this study, LPF does not induce any significant protection when injected intraperitoneally with LPS purified from *B. pertussis* and has no effect on OMP preparations from *N. gonorrhoeae* or *E. coli* containing moderate amounts of LPS.

It appears that, for a preparation to be protective against i.c. challenge with *B. pertussis*, it must satisfy two criteria. First, antigens are needed to stimulate the development of a protective immune response. Second, LPF is required to allow the immune response to develop at the site of infection by increasing the permeability of the blood-brain barrier to the antigens or antibodies or both and immune cells. In addition, an antibody response to LPF might also be required to achieve protection against the disease. It is not yet known whether the protective activity of gistic effect of residual biologically active LPF in the preparation or to the development of LPF antibodies which do not require access to the brain.

The relevance of the mouse i.c. potency assay has been questioned (17), although a good correlation has been found between the protective potency of vaccines in mice and children (10). However, this correlation was demonstrated with whole-cell vaccines, in which the LPF content of cells will be associated with other virulence factors, such as FHA, phase 1-specific OMP, and agglutinogens. Antigens that have been rigorously purified and are free from LPF do not appear to protect mice against i.c. challenge. Therefore, it is essential that candidate antigens for an acellular pertussis vaccine, including glutaraldehyde-treated LPF (14), should be assessed by a respiratory infection model, as has been done for FHA and OMP (2) and by in vitro antigen assays. Also, other antigens, omitted from a vaccine because they are not protective in the mouse i.c. protection test, should not be dismissed as being unimportant for protection of children.

ACKNOWLEDGMENTS

We thank D. C. Ellwood and L. A. E. Ashworth for helpful discussions and A. R. Blake, B. J. Eckersley, D. C. Hawkins, and G. R. G. Moody for invaluable technical assistance.

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