INFECTION AND IMMUNITY, July 1996, p. 2824–2828 Vol. 64, No. 7
0019-9567/96/$04.00+0

Structural and Immunological Characterization of *Burkholderia pseudomallei* O-Polysaccharide–Flagellin Protein Conjugates

PAUL J. BRETT AND DONALD E. WOODS*

Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary Health Sciences Centre, Calgary, Alberta, Canada T2N 4N1

Received 1 December 1995/Returned for modification 30 January 1996/Accepted 22 April 1996

The O-polysaccharide moiety of *Burkholderia pseudomallei* 319a lipopolysaccharide was covalently linked to flagellin protein isolated from the same strain. A glycoconjugate incorporating adipic acid dihydrazide as a spacer molecule elicited high-titer immunoglobulin G responses to both the protein and carbohydrate components of the construct. This immunoglobulin G was capable of protecting diabetic rats from challenge with a heterologous *B. pseudomallei* strain.

Burkholderia pseudomallei is the causative agent of melioidosis, a disease that afflicts both humans and animals (3, 16, 27). The manifestations of melioidosis are commonly represented by acute, subacute, and chronic illnesses, with the clinical indications of some stages often being mistaken for those associated with malaria, plague, pneumonia, and miliary tuberculosis (5, 16, 20, 23).

In previous studies, we have demonstrated that polyclonal and monoclonal antisera raised against *B. pseudomallei* flagellin proteins, lipopolysaccharide (LPS), and a tetanus toxoid glycoconjugate molecule provided passive protection against *B. pseudomallei* infections in animal model experiments (1, 2). It is clear, however, that an active vaccine preparation would be of most benefit to individuals at risk of acquiring *B. pseudomallei* infections. As spectroscopic and serological analyses of *B. pseudomallei* flagellin proteins and O-polysaccharide (PS) have shown that a high degree of conservation in these molecules, both on structural and immunological levels, is present in the majority of strains tested so far (1, 2, 15, 18), we propose that a conjugate molecule incorporating both flagellin protein and PS antigens would be a reasonable vaccine candidate for use in active immunization against melioidosis.

A flagellin-polysaccharide conjugate molecule possesses a variety of attributes considered to be desirable in the rational design of conjugate vaccines. The incorporation of two protective antigens from the same organism should enhance the immunological repertoire of the vaccine recipient while at the same time affording protection against strains that display serotypically distinct antigens of one of the two components. The conjugation of the PS to the carrier protein should also elicit an augmented immune response against the PS component while at the same time evoking desirable immunoglobulin (Ig) class switching events. Most importantly, the use of an active vaccine containing the PS portion of LPS but not the toxic component of LPS, namely, lipid A, conjugated to flagellin protein would obviate the toxic side effects of LPS but take advantage of the protective response to the PS moiety.

In this communication, we report the synthesis of a number of PS-flagellin conjugate molecules and the preliminary assessment of the immunological characteristics of such constructs for potential use in active immunoprophylaxis against *B. pseudomallei* infections.

Bacterial strains. *B. pseudomallei* strains were generously donated by D. A. B. Dance, Wellcome-Mahidol-Oxford Tropical Medicine Research Programme, Bangkok, Thailand. All stock cultures were maintained in a 10% skim milk suspension and stored at −70°C.

Flagellin isolation and purification. Flagellar filaments were obtained by modification of procedures outlined by Brett et al. (1).

Isolation and purification of LPS and PS. Lyophilized *B. pseudomallei* 319a cells were enzymatically treated as previously described by Johnson and Perry (14) prior to extraction with hot phenol as outlined by Westphal and Jann (25). The resulting purified LPS was shown to contain <1% nucleic acid or protein on a dry weight basis. Polysaccharide was obtained by solubilizing LPS in 3% acetic acid at 10 mg/ml and heating to 100°C in a sealed ampoule for 2 h (9). The PS was further purified on a Sephadex G-50 (Pharmacia Biotech) column (2.5 by 40 cm) with 100 mM sodium phosphate buffer (pH 7.4) as the eluting solvent. Fractions that emerged with a partition coefficient (K_{av}) of <0.45 ($K_{av} = V_e - V_0/V_t - V_0$) where V_e is the volume of distribution, V_0 is the voided volume, and V_t is the total bed volume) and which were positive for carbohydrate were pooled, dialyzed extensively against distilled water, and lyophilized.

Conjugate synthesis. The most immunogenic conjugate of those constructed in this study was synthesized by modification of methods previously described (2, 4). A solution of 20 mg of PS-adipic acid dihydrazide (ADH) in 5 ml of distilled water was acidified to pH 4.3 with 0.10 M HCl, and to this was added 2 ml of distilled water containing 2 mg of flagellin protein and 200 mg of 1-ethyl-3-(3-diethylaminopropyl) carbodiimide (Sigma Chemical Co.). The reaction mixture was stirred for 4 h, following which the product of the reaction was dialyzed against distilled water, clarified by low-speed centrifugation, and concentrated by ultrafiltration to approximately 3 ml. The conjugate mixture was applied to a Sepharose CL-4B gel column (2.5 by 40 cm) and eluted with a 100 mM sodium phosphate buffer (pH 7.4). Fractions demonstrating both protein and carbohydrate content were pooled, dialyzed against distilled water, and lyophilized. This glycoconjugate was named PS-ADH-FLA.

Coupling of PS to flagellin protein was also accomplished by activating the PS with either a combined reduction-oxidation procedure (13) or a simple one-step oxidation processes (6).

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary Health Sciences Centre, 3330 Hospital Dr., NW, Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-6885. Fax: (403) 270-2772.

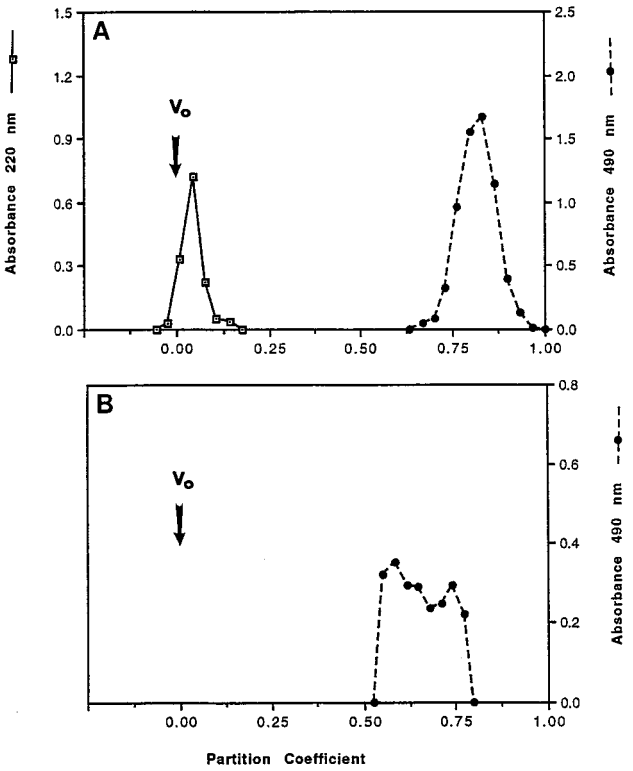

FIG. 1. Elution profiles of *B. pseudomallei* 319a flagellar protein, PS, and glycoconjugate molecules from Sepharose CL-4B gel permeation chromatography. (A) Elution profiles of flagellar protein and PS. (B) Elution profile of PS-ADH-FLA glycoconjugate reaction mixture as demonstrated by the presence of carbohydrate. V_0 represents the void volume of the column.

Following conjugation to protein by reductive amination, these glycoconjugates were termed (red/ox)PS-FLA and (ox)PS-FLA, respectively.

The elution profile of the PS-ADH-FLA reaction mixture demonstrates a partition coefficient shift in the carbohydrate-positive fractions compared with those of native PS eluted under the same conditions. All fractions testing positive for carbohydrate as assessed by the phenol-sulfuric acid method (10) from the coupling reaction were found to contain protein when assayed by the bicinchoninic acid method (Pierce Chemical Co., Rockford, Ill.). The absence of native precursor molecules in the column eluates was also demonstrated in the CL-4B fractions (Fig. 1). These results are all suggestive of a successful conjugation between the PS and the carrier protein molecules. After conjugation, the resulting conjugate molecules were heterogeneous, which is likely explained by the heterogeneity of size of the PS molecules employed in the conjugation procedures. Of the three conjugates synthesized for these studies, PS-ADH-FLA was the only construct to incorporate ADH as a spacer molecule between the coupled molecular species. The carbohydrate-to-protein ratio of this conjugate preparation was determined to be 0.88:0.12, while the average M_r of this construct compared with globular protein standards was 265,000. The (red/ox)PS-FLA and (ox)PS-FLA glycoconjugates did not require any spacer molecules for their synthesis, and the ratio of carbohydrates to proteins in both instances was calculated to be 0.86:0.14.

Antibody studies. Antibodies to the various glycoconjugates were prepared by administration of Freund's complete adjuvant (BRL Life Technologies, Inc., Gaithersburg, Md.) emulsified with filter-sterilized preparations of the individual conjugates in 10 mM phosphate-buffered saline, pH 7.0 (PBS). Rabbits (New Zealand White, 2 to 2.5 kg) each received an intramuscular injection of 0.50 ml of the emulsion in both their right and left hind thigh muscles. Each injection contained 100 μg of conjugate per ml. The rabbits received booster injections on days 14 and 28 post-primary immunization with Freund's incomplete adjuvant (BRL Life Technologies, Inc.) as the emulsifying agent, and test blood samples were taken at these points. Animals were exsanguinated by cardiac puncture under anesthesia at day 42, and serum samples were collected and stored at −70°C. The highest dilution of immune serum that reacted in an enzyme-linked immunosorbent assay (ELISA) against a specific antigen was expressed as the reciprocal value of that dilution.

The preimmune and immune serum titers of the rabbits immunized with the different conjugates were examined by ELISA and monitored for a period of 42 days post-primary immunization (Table 1). The PS-ADH-FLA serum clearly demonstrated an increase in IgG titers to both 319a LPS and flagellin proteins after primary immunization, as well as to the two subsequent booster doses. The (ox)PS-FLA conjugate preparation was the least immunogenic of the three studied. Its final titers for LPS and flagellin proteins were fivefold less than those of (red/ox)PS-FLA, with no booster response being observed for LPS between the tertiary dose and day 42. All further experiments requiring anticonjugate serum were conducted with the PS-ADH-FLA antiserum because of its high titers against both the LPS and flagellin proteins.

Rabbit polyclonal antiserum raised against the PS-ADH-FLA glycoconjugate preparation was shown to react strongly with both 319a flagellin protein and LPS, as demonstrated by Western blot (immunoblot) analysis (24) (Fig. 2). The immunostaining of the LPS lanes and the adjacent lanes, however, revealed a curious phenomenon. A distinct banding pattern was always found to reside in the wells adjacent to that which had been loaded, while the initially loaded lane displayed a more characteristic smear-like pattern (these results were obtained whether the sodium dodecyl sulfate [SDS]-polyacrylamide gels were loaded with the current running or not). Reactions of the flagellin and LPS samples with an anti-type II LPS-specific monoclonal antibody (MAb) demonstrated no reactivity with the purified flagellin proteins and reactivity with only one of the two distinct types of LPS patterns. Lanes adjacent to those loaded with LPS did not react with the MAb as they did with the antibodies directed against the conjugate, indicating that these lanes contained type I and not type II

TABLE 1. Evaluation of the immune responses to three *B. pseudomallei*-specific glycoconjugates in rabbits

Immunogen	IgG ELISA titers of immune serum to LPS or FLA on day[a]:					
	14		28		42	
	LPS	FLA[b]	LPS	FLA	LPS	FLA
PS-ADH-FLA	625	3,125	3,125	78,125	15,625	390,625
(red/ox)PS-FLA	ND[c]	ND	125	3,125	3,125	15,625
(ox)PS-FLA	125	125	625	625	625	3,125

[a] The ELISA titers of preimmune sera ($n = 2$) to both LPS and FLA obtained at the time of primary immunization (day 0) were <25. Immune serum samples were collected at 14-day intervals post-primary immunization. Rabbits were given booster injections on days 14 and 28 with Freund's incomplete adjuvant as the emulsifying agent.

[b] *B. pseudomallei* 319a flagellin protein.

[c] ND, not determined for this study.

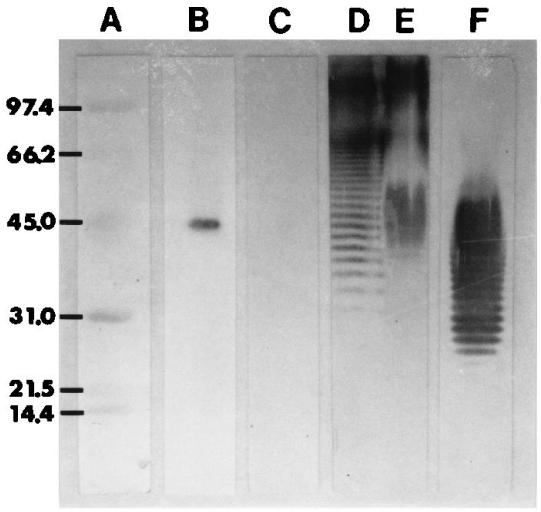

FIG. 2. Western blots of purified *B. pseudomallei* 319a flagellar protein and LPS. In lane A, standard protein markers (Bio-Rad Laboratories) were rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa). Lane B, purified flagellar protein (1 μg) reacted with a 1:2,000 dilution of PS-ADH-FLA polyclonal antiserum. Lane C, purified flagellar protein (10 μg) reacted with a 1:1,000 dilution of *B. pseudomallei*-specific anti-LPS MAb. Lane D, LPS material migrating from lane E. Lane E, purified LPS (10 μg). Lanes D and E reacted with a 1:500 dilution of anti-PS-ADH-FLA polyclonal antiserum. Lane F, purified LPS (3 μg) reacted with a 1:2,000 dilution of *B. pseudomallei*-specific anti-LPS MAb.

LPS. Such results suggest that the polyclonal conjugate antiserum is capable of recognizing both the type I and type II PS antigens of *B. pseudomallei* LPS. The reason why one type of LPS migrates from the wells is currently being investigated. Dot blot analysis of 38 *B. pseudomallei* strains, 319a flagellin proteins, LPS, PS, and the PS-ADH-FLA conjugate with the polyclonal rabbit serum demonstrated reactivity with all 38 strains and the 319a-derived samples. Analysis of the same samples with the LPS-specific MAb demonstrated reactivity with all but the flagellin proteins (data not shown).

To determine the functional activity of the anti-PS-ADH-FLA antiserum, we examined the ability of this antiserum to inhibit the motility of 38 *B. pseudomallei* strains (the results of experiments with strains 199a, 316c, 319a, 415e, and 420a are displayed in Fig. 3). Specificity was examined by testing the ability of this antiserum to inhibit the motility of *P. aeruginosa* PAO (Fig. 3). The PS-ADH-FLA antiserum at a 1/100 dilution was shown to inhibit or attenuate the motility of 38 of 38 strains tested and was comparable in efficacy to a 1/100 dilution of anti-319a flagellin antiserum which has previously been shown to inhibit the motility of 64 of 65 *B. pseudomallei* strains (1). Neither of the two antisera which inhibited the *B. pseudomallei* strains was able to inhibit the motility of *P. aeruginosa* PAO. The anti-LPS MAb was ineffective in inhibiting the motility of either of the two bacterial species. These results demonstrate that the PS-ADH-FLA antiserum is both functional and specific.

An Affi-Gel Protein A MAPS II kit (Bio-Rad Laboratories) was used to isolate the IgG fraction of the crude rabbit polyclonal antiserum produced against PS-ADH-FLA. The purified immunoglobulin fraction was utilized for passive immunoprotective studies in an animal model of *B. pseudomallei* infection (26). Briefly, 100 Sprague-Dawley rats (approximately 30 g; Charles River, Quebec, Canada) were made diabetic by streptozotocin administration on each of two consecutive days. One-half of the animals served as unimmunized controls and received 100 μl of PBS-bovine serum albumin (BSA) (15 mg/ml) by intravenous injection in their tail veins. The remaining 50 animals were passively immunized by injection of 100 μl of PBS containing 1.5 mg of purified IgG anti-PS-ADH-FLA. Ten animals in each of the control and immunized groups were inoculated intraperitoneally with one of five different doses of *B. pseudomallei* 316c: 3×10^3, 3×10^4, 3×10^5, 3×10^6, or 3×10^7. Animals were monitored carefully for eight consecutive days for signs of mortality. Fifty percent lethal doses (LD_{50}s) were calculated according to the method of Reed and Muench (19). This animal model is not amenable to active immunization studies, as active immunization would require a minimum of 4 weeks. At this time, the animals would be adults, and adult diabetic rats are not susceptible to intraperitoneal challenge with *B. pseudomallei* (unpublished observations).

Of the rats in the PBS control groups, only 20% were still alive after the eighth day, whereas the immunized groups displayed a 60% survival rate over the same time frame. The most significant results were found in the 3×10^3 and the 3×10^4 innoculum groups. The calculated LD_{50} for the control group was 7.54×10^3, while that for the immunized group was 8.89×10^5 (Table 2). Previous studies have defined *B. pseudomallei* LPS and flagellin protein as protective antigens (1, 2). Thus, we constructed a number of PS-flagellin protein glycoconjugates in order to assess whether the combination of these protective antigens would be useful for prophylaxis against melioidosis. The size of the conjugate molecule appears to greatly influence the immunogenicity of the preparation, so a variety of PS chain lengths were incorporated into the glycoconjugates in order to achieve this criterion (7, 8, 12). This incorporation enabled the larger PS fragments to increase the sizes of the conjugate molecules while at the same time presenting conformationally stabilized epitopes which appear to be critical in the design of a successful vaccine (12). The smaller oligosaccharide fragments would in turn act to elicit immunoglobulin responses against terminal epitopes of the carbohydrate molecules.

The choice of the carrier protein was based upon previous passive immunization studies that demonstrated the protective capacities of antisera raised against purified flagellin preparations (1). By utilizing these proteins as carrier molecules, the

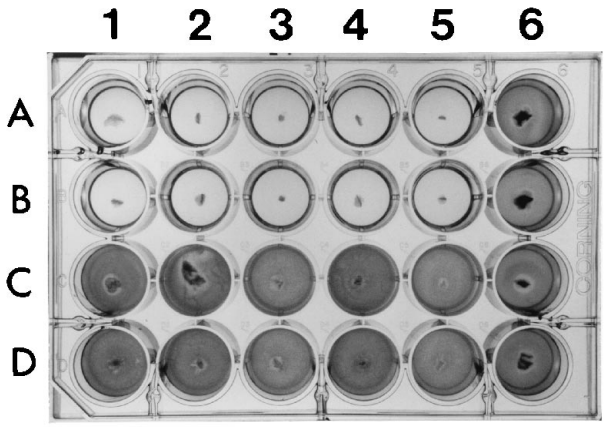

FIG. 3. Motility inhibition assessment of selected *B. pseudomallei* strains and *P. aeruginosa* PAO reacted with various *B. pseudomallei*-specific antiserum samples. Row A contains 1:100 dilutions of anti-319a flagellum-specific polyclonal antiserum. Row B contains 1:100 dilutions of anti-PS-ADH-FLA polyclonal antiserum, while row C contains 1:100 dilutions of *B. pseudomallei*-specific anti-LPS MAb. Row D is a control lane with 1:100 dilutions of PBS. Lanes were inoculated as follows: 1, *B. pseudomallei* 199a; 2, *B. pseudomallei* 316c; 3, *B. pseudomallei* 319a; 4, *B. pseudomallei* 415e; 5, *B. pseudomallei* 420a; and 6, *P. aeruginosa* PAO.

TABLE 2. Passive immunoprotection by anti-319a PS-ADH-FLA IgG in diabetic rats challenged with a heterologous *B. pseudomallei* strain[a]

Inoculum size (CFU)	Mortality of diabetic rats of group ($n = 10$):	
	Control (PBS-BSA IV)	Immunized (IgG IV)
3×10^3	6	0
3×10^4	7	0
3×10^5	7	5
3×10^6	10	6
3×10^7	10	9

[a] Groups of diabetic rats (10 rats in each group) were inoculated with 10-fold dilutions of *B. pseudomallei* 316c ranging from 3×10^3 to 3×10^7 CFU per rat and administered IgG or PBS-BSA IV. The LD_{50}, calculated by the method of Reed and Muench (19), for immunized rats was 8.89×10^5. That of the control group was 7.54×10^3, which was significantly lower than that of the immunized group ($P < 0.001$ by chi-square analysis).

conjugates display not one but two protective antigens in the same molecule, a desirable attribute for a vaccine to possess since it decreases the likelihood that the organism may evade the humoral components of the host response because of the loss or a mutation in one of the two protective antigens. Also, the use of a flagellin protein carrier would address the concern of generating a state of immunotolerance in the vaccine recipient (11, 17, 21, 22).

The major focus of these studies was to assess the impact of the various coupling procedures on the immunogenicities of the glycoconjugate molecules. The results of assays to determine rabbit immune serum titers suggest that the method of PS activation and derivatization and the covalent linkage of the modified PS fragments to the carrier proteins play essential roles in determining the immunological profiles of the conjugate molecules. Since identical PS fragment sizes and carrier proteins were used in all three conjugation experiments, these molecules could be eliminated as sources of deviation among the immune titers. Whether one or more of the steps required for the coupling procedures were responsible for these differences is yet to be determined. There is no doubt, however, that the PS-ADH-FLA construct was superior in its ability to elicit high-titer sera to both LPS and flagellin protein molecules, compared with (ox)PS-FLA and (red/ox)PS-FLA, and that the anamnestic response observed following the booster doses suggests a conversion of the T-cell-independent PS fragments into T-cell-dependent antigens. The presence of two unique LPS banding patterns recognized by the PS-ADH-FLA antiserum suggests that both the type I and II PS antigens are recognized by the immune serum (15, 18). The more prominent banding profile which was a result of antigen migration from a loaded well to adjacent wells (even when loaded with an electrophoretic current) is most likely the type I antigen. This conclusion is substantiated by the inability of the type II-specific anti-LPS MAb to react with this particular component. The reason why only this specific antigen migrates from the well of the SDS-polyacrylamide gels as it does has yet to be fully explained.

Dot blot analysis of 38 *B. pseudomallei* strains with the conjugate serum demonstrated reactivity with all 38 strains and suggests that the immune serum should be effective for protecting against numerous *B. pseudomallei* strain challenges. The level of protection is less than those previously determined for a 304b anti-LPS MAb and 319a flagellin-specific polyclonal antiserum (1, 2), but a number of factors may account for this anomaly. The absence of conformational epitopes in the conjugate preparations may explain a loss in the protective capacity of the conjugate antiserum compared with those of purified flagellin proteins. The 2.7-fold difference in antibody titers against the flagellin proteins, as demonstrated by the PS-ADH-FLA and 319a-specific flagellin antisera, would also be expected to play a significant role in the protective capacities of these antisera.

In this communication, we have demonstrated that the conjugation of *B. pseudomallei* PS to flagellin proteins results in an immunogenic glycoconjugate preparation. The construct has been shown to retain antigen-specific and protective epitopes of the precursor molecules as well as demonstrating secondary Ig responses to the normally T-cell-independent PS antigens. This finding suggests that a successful conversion of the PS antigens to T-cell-dependent antigens has occurred. Since *B. pseudomallei* strains have been shown to present both the type I and II PS antigens (all except 824a, which displays only the type I antigen) and the flagellin proteins appear to be highly conserved on an immunological basis (1, 18), we expect the PS-ADH-FLA conjugate molecule to afford protection against the majority of *B. pseudomallei* strains that we will encounter in our future studies.

This work was supported by the Canadian Bacterial Diseases Network of Centres of Excellence and by the Canadian Department of National Defense, contract number W7702-5-R512/01-XSG.

REFERENCES

1. **Brett, P. J., C. W. Mah, and D. E. Woods.** 1994. Isolation and characterization of *Pseudomonas pseudomallei* flagellin proteins. Infect. Immun. **62:** 1914–1919.
2. **Bryan, L. E., S. Wong, D. E. Woods, D. A. B. Dance, and W. Chaowagul.** 1994. Passive protection of diabetic rats with antisera specific for the polysaccharide portion of the lipopolysaccharide isolated from *Pseudomonas pseudomallei*. Can. J. Infect. Dis. **5:**170–178.
3. **Chaowagul, W., N. J. White, D. A. B. Dance, Y. Wattanagoon, P. Naigowit, T. M. E. Davis, S. Looareesuwan, and N. Pitakwhatchara.** 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J. Infect. Dis. **159:**890–899.
4. **Cryz, S. J., Jr., J. C. Sadoff, E. Furer, and R. Germanier.** 1986. *Pseudomonas aeruginosa* polysaccharide-tetanus toxoid conjugate vaccine: safety and immunogenicity in humans. J. Infect. Dis. **154:**682–688.
5. **Dar, L., R. Thakur, and V. S. Dar.** 1994. India: is it plague? Lancet **344:**1359.
6. **Dean, P. G. D., W. S. Johnson, and F. A. Middle.** 1987. Affinity chromatography: a practical approach, p. 46–47. IRL Press, Oxford.
7. **Dintzis, R. Z., M. H. Middleton, and H. M. Dintzis.** 1983. Studies on the immunogenicity and tolerance of T-independent antigens. J. Immunol. **131:** 2196–2203.
8. **Dintzis, R. Z., M. H. Middleton, and H. M. Dintzis.** 1985. Inhibition of anti-DNP antibody formation by high doses of DNP-polysaccharide molecules: effect of hapten density and valence. J. Immunol. **143:**423–427.
9. **Drewry, T. D., G. W. Gray, and S. G. Wilkinson.** 1971. Release of ethanolamine pyrophosphate during mild acid hydrolysis of the lipopolysaccharide of *Pseudomonas aeruginosa*. Eur. J. Biochem. **21:**400–403.
10. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
11. **Herzenberg, L. A., T. Tokuhisa, and L. A. Herzenberg.** 1980. Carrier priming leads to hapten-specific suppression. Nature (London) **285:**665–666.
12. **Jennings, H.** 1992. Further approaches for optimizing polysaccharide-protein conjugate vaccines for prevention of invasive bacterial disease. J. Infect. Dis. **165:**S156–S159.
13. **Jennings, H., and C. Lugowski.** 1981. Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J. Immunol. **127:** 1011–1018.
14. **Johnson, K. G., and M. B. Perry.** 1975. Improved techniques for the preparation of bacterial lipopolysaccharides. Can. J. Microbiol. **22:**29–34.
15. **Knirel, Y. A., N. A. Paramonov, A. S. Shashkov, N. K. Kochetkov, R. G. Yarullin, S. M. Farber, and V. I. Efermenko.** 1992. Structure of the polysaccharide chains of *Pseudomonas pseudomallei* lipopolysaccharides. Carbohydr. Res. **233:**185–193.
16. **Leelarasamee, A., and S. Bovornkitti.** 1989. Melioidosis: review and update. Rev. Infect. Dis. **11:**413–425.
17. **Peeters, C. C. A. M., A.-M. Tenbergen-Meekes, J. T. Poolman, M. Beurret, B. J. M. Zegers, and G. T. Rijkers.** 1991. Effect of carrier priming on immunogenicity of saccharide-protein conjugate vaccines. Infect. Immun. **59:**3504–3510.

18. **Perry, M. B., L. L. MacLean, T. Schollaardt, L. E. Bryan, and M. Ho.** 1995. Structural characterization of the lipopolysaccharide O-antigens of *Burkholderia pseudomallei*. Infect. Immun. **63:**3348–3352.
19. **Reed, L. J., and H. Muench.** 1937. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
20. **Sanford, J. P.** 1985. Pseudomonas species (including melioidosis and glanders), p. 1250–1254. *In* G. L. Mandell, R. G. Douglas, and J. E. Bennet (ed.), Practice and principles of infectious diseases. Churchill Livingstone, New York.
21. **Shutze, M. P., E. Deriaud, G. Przewlocki, and C. Leclerc.** 1989. Carrier-induced epitopic suppression is initiated through clonal dominance. J. Immunol. **142:**2635–2640.
22. **Shutze, M. P., C. Leclerc, M. Jolivet, F. Audibert, and L. Chedid.** 1985. Carrier-induced epitope suppression, a major issue for synthetic vaccines. J. Immunol. **135:**2319–2322.
23. **Smith, C. J., J. C. Allen, M. N. Embi, O. Othman, N. Ratzak, and G. Ismail.** 1987. Human melioidosis: an emerging medical problem. MIRCEN J. **3:**343–366.
24. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **74:**4350–4354.
25. **Westphal, O., and K. Jann.** 1965. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **25:**83–91.
26. **Woods, D. E., A. L. Jones, and P. J. Hill.** 1993. Interaction of insulin with *Pseudomonas pseudomallei*. Infect. Immun. **61:**4045–4050.
27. **Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hota, Y. Hashimoto, T. Ezaki, and M. Arakawa.** 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36:**1251–1275.

Editor: R. E. McCallum