Polymyxin B Inactivation of Lipopolysaccharide in Vaccines of Gram-Negative Bacteria

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Endotoxin activity in suspensions of *Bordetella pertussis*, *Escherichia coli*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* was often markedly decreased by polymyxin B. Polymyxin B treatment may be a means to reduce inflammatory reactivity of lipopolysaccharide in vaccines of gram-negative bacteria.

A simple means to reduce the inflammatory activity caused by the lipid A moiety of lipopolysaccharide (LPS) in vaccines of gram-negative bacteria would be useful. Polymyxin B binds to enterobacterial LPS at its lipid A-ketodeoxyoctonate region (2, 24), blocking many of its biological effects in vitro (3, 24, 25, 27) and its toxic effects in vivo (8-10, 15, 28, 30-32). *Pseudomonas* (16), *Haemophilus* (14), and *Neisseria* (7) LPS appear to be similarly susceptible. The *Limulus* gelation assay appears to reflect toxic activities of LPS in laboratory animals (6, 12, 23, 36) and man (21), is relatively specific for LPS (36), and has previously been used as a means to demonstrate detoxification of LPS by polymyxin B in vitro (7). Since whole bacterial cells may offer certain advantages as vaccines, the ability of polymyxin B to inactivate the *Limulus* gelation activity of several genera of gram-negative bacilli was studied.

Escherichia coli J5 was provided by A. I. Braude. Diphtheria-tetanus-pertussis (DTP) vaccines were purchased from Wyeth Laboratories (lot 66601) and Lederle Laboratories (lot NDC0005). *Haemophilus influenzae* type b and *Pseudomonas aeruginosa* were isolated locally from spinal fluid and blood, respectively. *Bacteroides vulgatus* strain 13079 was provided by V. R. Dowell. Boivin *E. coli* 026B6 LPS standard (Difco Laboratories) and polymyxin B (lot 96134; Pfizer, Inc.) were purchased commercially. Lyophilized *Limulus* amoebocyte lysate (F. Pearson, Travenol Laboratories) (26) was suspended in normal saline. The assay, performed as described previously (29), consistently detected 0.3 to 1.0 ng of standard endotoxin per ml.

DTP vaccine, as provided by the manufacturer, was held at 4°C until use. For detoxification experiments, 9 volumes of DTP vaccine

were mixed with 1 volume of water or with 1 volume of polymyxin B solution to give final concentrations of 100 or 1,000 µg of polymyxin B per ml of suspension. The mixtures were incubated overnight at room temperature with gentle agitation and then the endotoxin activity was determined by testing serial dilutions in the *Limulus* assay, standardized with 026B6 LPS. In some instances, incubation of bacterial suspensions with polymyxin B was carried out for 30 min or overnight at 37°C.

E. coli J5 was prepared from a single colony grown overnight in tryptic soy broth (Difco Laboratories). The bacteria were washed three times in normal saline, heated at 100°C for 2.5 h, and again washed three times. If not used immediately, the bacteria were stored at 4°C and again washed three times just before being tested in detoxification experiments. Bacterial counts were determined in a Petroff-Hauser chamber.

H. influenzae was isolated on chocolate agar plates, grown overnight in tryptic soy broth with supplement C (Difco Laboratories), washed three times, suspended in 0.5% Formalin for 3 days, washed three times in normal saline, and used immediately or held at 4°C until used. *P. aeruginosa* was isolated on sheep blood agar plates, grown overnight in tryptic soy broth, and handled as described above for *H. influenzae*. A single colony of *B. vulgatus* was streaked for confluent growth on several brucella blood agar plates. After 48 to 72 h of anaerobic incubation, the bacteria were removed from the plates, washed three times, and handled as described above for *H. influenzae*. All stored bacterial suspensions except DTP were again washed immediately before beginning detoxification experiments. In some instances, the final wash supernatant was tested for endotoxin activity in the *Limulus* assay and was always found to contain

activity less than or equal to that of the resuspended bacteria.

Buffers were not utilized for the experiments; all reactions were carried out at pH 7.0 to 7.4 by first adjusting the reagents with small amounts of dilute NaOH or HCl if necessary.

Polymyxin B activity was determined by adding 10 μ l of test material to filter paper disks placed on Mueller-Hinton agar (Scott) plates seeded with *P. aeruginosa* ATCC strain 27853. Inhibition zone diameters were compared with those produced by untreated polymyxin B in serial twofold dilution. The assay could detect a minimum of 100 μ g of polymyxin B per ml.

Results of detoxification experiments are shown in Table 1. In most instances, endotoxin activity associated with *Bordetella pertussis*, *E. coli* J5, *P. aeruginosa*, and *H. influenzae* was sharply reduced by both 100 and 1,000 μ g of polymyxin B per ml. A considerable degree of variation in the level of toxic activity of bacterial strains and in the degree of susceptibility to polymyxin B was encountered. Detoxification was altered little, if any, by reducing incubation time to 30 min or by increasing incubation temperature to 37°C.

B. vulgatus was tested as a control strain, since the *Limulus* activity of a *Bacteroides* preparation had previously been demonstrated to resist inactivation by polymyxin (7). *B. vulgatus* activity was not reduced in either of two trials when either 100 or 1,000 μ g of polymyxin B per ml was used. This indicates that polymyxin B in the concentrations used in these experiments does not inhibit the *Limulus* assay.

In several experiments (not shown), lyophilized *Limulus* lysate was reconstituted in 40%

normal unheated human serum instead of saline, to reduce the sensitivity of the test. In these experiments, endotoxin inactivation by polymyxin B could be demonstrated to be as effective as when the lysate was dissolved in water. Little or no dilution of the vaccine-polymyxin B mixtures was needed to show the effect of polymyxin B in this system, indicating that the inactivation observed was not a function of the high dilutions required when more sensitive lysate was used.

The activity of polymyxin B remaining in the bacteria-polymyxin B mixtures was assessed by disk diffusion assay in two instances. Neither 3.8×10^8 *E. coli* J5 per ml nor DTP vaccine detectably altered the activity of 1,000 μ g of polymyxin B per ml in the suspension, suggesting that polymyxin B was present in excess.

This study demonstrates the possibility that toxic LPS in vaccines of gram-negative bacteria might be effectively neutralized by pretreatment with polymyxin B. With the present study, *B. pertussis* and *P. aeruginosa* may be tentatively added to the list of organisms known to produce LPS which can be detoxified with polymyxin B. The LPS activity of *B. vulgatus* was found to resist polymyxin B neutralization, as had been shown earlier for *B. fragilis* (7).

It is likely that polymyxin B acts both upon LPS within the bacterial membrane and upon LPS-containing complexes released during the course of incubation. Wash fluids contained LPS activity near or equal to those of the resuspended, washed bacteria, and it is likely that the release of LPS occurs continuously. Therefore, despite the fact that the bacteria may have been washed before testing, it remains unclear whether polymyxin B action upon bacilli or the released LPS complexes contributed the most to the effect observed. Kreeftenberg and co-workers (20) observed that the supernatant of centrifuged typhoid vaccine contained more LPS activity than the sedimented bacilli, for example, and we would expect this to be true of other vaccines of whole cell gram-negative bacteria as well.

Vaccines of whole cell bacteria have the potential advantages that surface antigens are more likely to be presented in a natural orientation and that the adjuvant effects seemingly inherent in complex, particulate vaccines (18, 38) can be maintained. In addition, purification procedures potentially causing structural alterations of important immunogens can be avoided. However, it remains uncertain whether the immunogenic properties of polymyxin-treated bacteria remain intact. Jacobs and Morrison (17) reported that the in vitro specific immune response of cultured spleen cells to purified LPS was unaltered or even enhanced by treatment of

TABLE 1. Effect of polymyxin B upon LPS activity of gram-negative bacteria in the *Limulus* gelation test

Bacteria tested	Bacteria (per ml) ^c	Relative LPS activity (μ g/ml) ^b	Log ₁₀ reduction after treatment with polymyxin B at:	
			100 μ g/ml	1,000 μ g/ml
DTP vaccine (Lederle)	ND ^a	3,000	2	3
DTP vaccine (Wyeth)	ND	100	ND	6
<i>E. coli</i> J5, heat killed	2.6×10^6	10	0	3
<i>P. aeruginosa</i> , formalinized	1.2×10^7	0.3	4	4
<i>H. influenzae</i> , formalinized	3.0×10^6	1	3	3
<i>B. vulgatus</i> , formalinized	3.0×10^6	3	0	0

^a Count as determined in a counting chamber.

^b Compared with 026B6 *E. coli* Boivin LPS standard.

^c ND, Not determined.

LPS with polymyxin B. Smith et al. (33) also found that polymyxin B enhanced the immunogenicity of hapten-conjugated LPS, but only when high, previously toxic, LPS concentrations were used. In the only *in vivo* study we have been able to identify, Dufer and co-workers (11) reported reduction in LPS bioactivity by polymyxin B pretreatment without concomitant reduction in LPS immunogenicity. We have found no studies on the immunogenicity of polymyxin-treated whole bacteria. It has been shown, however, that the ability of several *Serratia marcescens* outer membrane components to bind preformed antibody in an immunoelectrophoresis system was partially lost after treatment of either whole bacteria or isolated membranes with high concentrations of polymyxin B similar to those used in the present study (4, 37). It is also known that polymyxin binds to bacterial surface components other than LPS, most notably phospholipids (13, 22, 35).

Bacterial toxins other than LPS (19) would likely not be affected by polymyxin B, and polymyxin B itself has potential toxicity (1, 5, 15), although the levels employed in the present study would represent only a fraction of ordinarily recommended therapeutic doses of polymyxin B.

Despite the sometimes striking reduction of whole bacterial LPS activity demonstrated in the present study, a clear dose-response relationship could not be demonstrated. The effects of both the relative activity of bacterial suspensions in the *Limulus* assay and the amount of polymyxin B proved to be quite variable during the course of these studies. Undoubtedly, this was due to the complex and unstable nature of killed bacterial suspensions. Further, concentrations of polymyxin B far in excess of amounts predicted from known optimal polymyxin B-LPS ratios (7, 11, 24) and the measured activity of the bacterial suspensions in *limulus* assay were required. Thus, the optimal conditions for polymyxin B treatment of vaccines of whole bacteria have yet to be established. Manipulation of culture media, particularly with regard to cation concentration, may enhance polymyxin B susceptibility (34). Polymyxin B-treated outer membrane vaccine preparations might also be usefully studied. Finally, most data on the effectiveness of the polymyxins has been based on bactericidal activity. A determination of optimal structures to effect LPS detoxification could lead to development of pharmaceuticals useful in treating endotoxemia and in the development of vaccines of gram-negative bacteria.

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