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Polymyxin B Use Does Not Ensure Endotoxin-Free Solution

Matthew J. Kluger, Rebecca Singer and Steven M. Eiger

Department of Physiology, The University of Michigan Medical School, Ann Arbor, MI 48109, U.S.A.

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Polymyxin B is often added to in vitro samples to 'ensure' that endotoxin activity is removed. We present data, from the standard rabbit pyrogen test and the *Limulus* amebocyte lysate assay, that polymyxin B bound to a gel support will bind some, *but not all*, endotoxin. These data, in conjunction with previously published data by Morrison and Curry (1979), indicate that those studies that have relied on polymyxin B to inactivate endotoxin must be re-evaluated.

Key words: *polymyxin B – endotoxin – fever – lipid A-associated protein (LAP) – Limulus amebocyte lysate assay*

Introduction

Polymyxin B is a small peptide derived from *Bacillus polymyxa* that has widespread antibiotic activity against Gram-negative bacteria (Sande and Mandell, 1980). Morrison and Jacobs (1976) have shown that polymyxin B binds to the lipid A region of bacterial endotoxins. Since the lipid A portion of endotoxin is generally considered the active site of the molecule, both for its toxic and beneficial effects (Nowotny, 1983), within a short time many investigators were reporting that polymyxin B could be used to *reduce* the activity of endotoxin both in vitro and in vivo (Van Miert and Van Duin, 1978; From et al., 1979; Cooperstock and Riegle, 1981). Morrison et al. (1976) have shown that the lipid A-associated protein (LAP) that is present in endotoxin inhibits the binding of polymyxin B to the endotoxin (Morrison and Curry, 1979). As a result, one might surmise that the effectiveness of using polymyxin B to remove endotoxins from solution would be of limited value. Despite the above data, within the past 2 or 3 years many investigators have suggested that polymyxin B, either free or bound to a gel support, can be used to 'ensure' that a solution is free of endotoxins (for example, see Duff and Atkins, 1982; Dinarello, 1983; Issekutz, 1983; Dinarello et al., 1984).

In this study we report that, based on rabbit pyrogen assays and on *Limulus* amebocyte lysate assays, polymyxin B bound to agarose beads effectively removes some, *but not all*, endotoxin from solution. As a result of our data, plus the earlier

study by Morrison and Curry (1979) showing that free polymyxin will not bind to endotoxin, extreme caution should be used before claiming a solution endotoxin-free based on the addition of polymyxin B.

Materials and Methods

Polymyxin B sulfate (Sigma) was bound to Affi-Gel 10 based on the method of LaPorte et al. (1977). Prior to use, the gel was washed with 300 ml isopropanol (4°C) and 300 ml deionized water (4°C). Each gram of polymyxin B was mixed with 100 ml of 100 mM $NaHCO_3$ (pH = 8.3) and stirred at 4°C until the polymyxin B was completely dissolved. Affi-Gel 10 was then added and allowed to couple with the polymyxin B for 4 h at 4°C with mild agitation. The concentration of polymyxin B used was 15 times that of the active ester *N*-succinimide spacer arm (247.4 mg of polymyxin B/ml gel, approximately 1,500,000 U/ml gel, dissolved in 100 ml buffer), as recommended by Bio-Rad. The slurry was then applied to an Econo-column (0.5×20 cm, no. 737-0242; Bio-Rad, St. Louis, MO) and the buffer eluted. Additional slurry was added until the bed volume contained approximately 4 ml of gel. Pyrogen-free 0.9% sodium chloride (saline) was then dripped through the column by continuous siphon, and the columns were stored at 4°C. When absorbance analysis of the eluent at 280 nm equalled 0 relative to the standard, it was assumed that no further polymyxin B was leaching off the column, and test samples were then run through the column at 4°C and collected in sterile polystyrene plastic tubes. Test samples consisting of varying concentrations of endotoxins were applied to the column and eluted by gravity. A total of 20 ml of sample was passed through the column and the second 10 ml of eluent was saved for assay for endotoxin.

The endotoxins used in these studies were:

(a) *Escherichia coli* 0111 : B4, phenol-extracted (Sigma no. L2630);

(b) *E. coli* 0111 : B4, phenol-extracted (Difco no. 3122-25-0);

(c) *Salmonella typhimurium*, phenol-extracted (Sigma no. L6511);

(d) *E. coli* strain K-37 provided by Dr. David Friedman, Department of Microbiology and Immunology, The University of Michigan Medical School, phenol-extracted.

(e) In addition to the above endotoxins, we tested whether the columns would remove a pyrogenic dose of endotoxin derived from heat-killed bacteria. *Salmonella typhimurium* (LT-2 wild type from ATCC) bacteria were grown on sheep blood-agar plates, washed in sterile pyrogen-free saline, and heat-killed. The concentration of dead bacteria was determined to be 3×10^9 bacteria/ml by comparison with Bacto McFarland Barium Sulfate Turbidity Standards (Difco). This suspension was filtered through a 0.22 μM filter and tested for the presence of endotoxin, both pre- and post-column. In addition, a portion of this suspension was centrifuged and the cell-free supernatant was saved and tested for endotoxin, pre- and post-column.

(f) Had the columns been 100% effective in removing endotoxins from solution, these columns could be used to remove an unspecified mixture of endotoxin. To test this hypothesis we collected urine, which is known to contain the breakdown

products from a variety of bacteria, from healthy male donors. Both whole urine and urine filtered through 0.22 μM filters were tested pre- and post-column for the presence of endotoxins.

Endotoxin activity was assayed using 2 methods: the rabbit pyrogen test, and the *Limulus* lysate assay (Sigma). The pyrogen test used male New Zealand White rabbits (*Oryctolagus cuniculus*), previously acclimated to standard rabbit restrainers. Each animal was placed in a restrainer in an ambient temperature of 15°C. Rectal temperatures were recorded every 10 min using a 32-channel Digistrip III datalogger (Kaye Inst.). After reaching a steady state rectal temperature (rectal temperature not varying more than ± 0.1°C) for at least 30 min, 1.0 ml of solution to be tested was injected i.v. into each rabbit via the marginal ear vein. Average temperature changes for the 4-h period following injection were determined for each solution tested. The *Limulus* assay used lysate derived from the amebocytes of *Limulus polyphemus* to detect the presence of endotoxin. A cloudy gel forms when the lysate is exposed to endotoxin. In each assay both negative (pyrogen-free water) and positive (known quantity of endotoxin) controls were used. Briefly the protocol was as follows: 0.1 ml of experimental solution (diluted to a concentration within the assay's determined range of sensitivity) and 0.1 ml of lysate were combined in a polystyrene tube, and incubated for 1 h at 37°C. At this time, the sample was judged positive for endotoxin when gelation occurred, or negative for endotoxin when no gelation occurred.

In addition to determining which endotoxins would be effectively removed by these columns, we determined the maximum amount of endotoxin bound/ml of gel for the *E. coli* endotoxin from Sigma. Ten μg endotoxin/ml of saline was run through columns maintained at both room temperature (approx. 22°C) and at 4°C. The *E. coli* solution was run through the columns, and samples collected and tested for the presence of endotoxin by the rabbit pyrogen assay. In a separate experiment, we also determined whether the columns would remove a 10-fold higher concentration of endotoxin from solution. Ten ml of 100 μg of *E. coli* endotoxin from Sigma/ml of saline was passed through the column and the removal of endotoxin was determined by the rabbit pyrogen test.

Data were analyzed by Student's *t*-test and analysis of variance using the Michigan Interactive Data Analysis System (MIDAS) at the University of Michigan.

Results and Discussion

The effects of passing *E. coli* endotoxin (0111 : B4) from Sigma and Difco through the columns on the pyrogenicity of the eluent is shown in Fig. 1A and B. The endotoxin from Sigma was completely removed by passage through the columns. Although the *E. coli* endotoxin from Difco was of the same serotype as that from Sigma and was extracted by the same method (phenol method of Westphal), the endotoxin from Difco was only partially removed. A summary of the *average* fevers developed over the 4-h post-injection period is shown in Fig. 2. The columns effectively removed *E. coli* endotoxin (0111 : B4, Sigma), as well as *S. typhimurium*

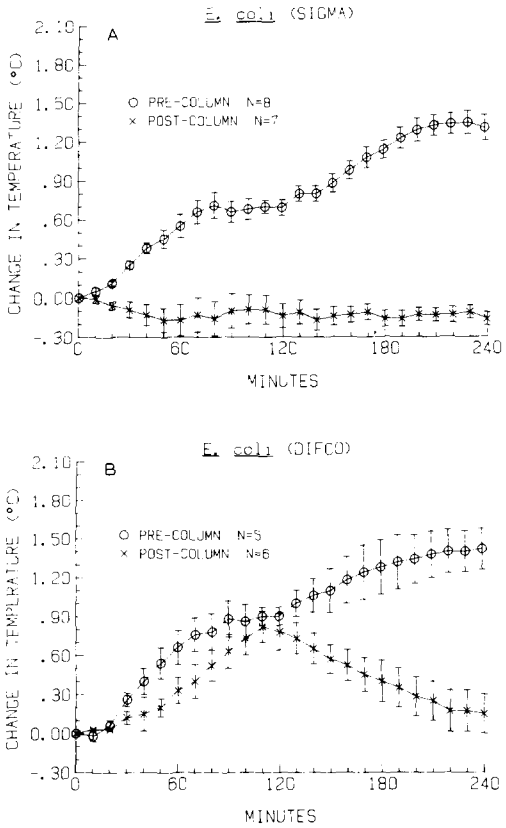

Fig. 1. Effect of injecting (1 ml, intravenously) pre- and post-polymyxin-Affi-Gel column solutions on the mean (±1 SE) change in rectal temperature of the New Zealand White rabbit. Pre-column solutions contained 10 μg/ml endotoxin. All injections occurred at time = 0 min. N, sample size. A: *E. coli* endotoxin (0111 : B4), Sigma. B: *E. coli* endotoxin (0111 : B4), Difco.

endotoxin (Sigma) and *E. coli* endotoxin (K-37). While *S. typhimurium* endotoxin from Sigma was effectively inactivated by passage through the columns, the pyrogenic activity of the filtrate of heat-killed *S. typhimurium* bacteria was only partially removed by the column. Interestingly, passage of the filtrate of heat-killed *S. typhimurium* through the column a second time resulted in no further removal of endotoxin, based on the pyrogen test (Fig. 3).

The pre- and post-column samples were also assayed using the *Limulus* lysate assay. As expected, all pre-column samples had considerable amounts of endotoxin. Post-column samples from *E. coli* endotoxin (Sigma), *S. typhimurium* endotoxin (Sigma) and *E. coli* endotoxin (K-37) were relatively free of endotoxin; eluents from *E. coli* (Difco), heat-killed *S. typhimurium* (both before and after centrifugation) and from urine (both nonfiltered and filtered) contained significant amounts of endotoxin and were pyrogenic (Table I). The failure of the columns to effectively remove endotoxin from *E. coli* (Difco), heat-killed *S. typhimurium* and urine samples, may

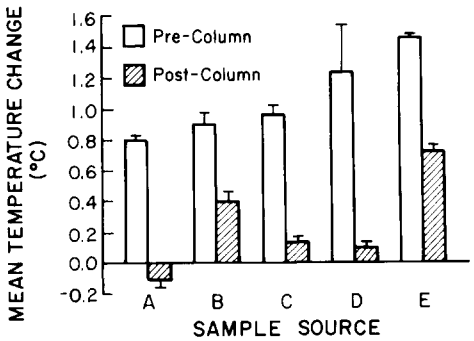

Fig. 2. Effect of injecting (1 ml, intravenously) pre- and post-polymyxin-Affi-Gel column solutions on the average 4-h (± 1 SE) change in rectal temperature of the New Zealand White rabbit. The pre-column solutions from *E. coli* (K-37) and the filtrate of heat-killed *S. typhimurium* contained 0.5 μg/ml endotoxin. All other pre-column solutions contained 10 μg/ml endotoxin. A: *E. coli* endotoxin (0111 : B4) (Sigma); sample size 8 pre-column, 7 post-column. B: *E. coli* endotoxin (0111 : B4) (Difco); sample size 5 pre-column, 6 post-column. C: *E. coli* endotoxin, phenol-extracted (K-37); sample size 4 pre-column, 6 post-column. D: *S. typhimurium* endotoxin (Sigma no. L6511); sample size 3 pre-column and 3 post-column. E: Filtrate of heat-killed *S. typhimurium* (LT-2 wild type from ATCC); sample size 3 pre-column and 3 post-column.

be related to the presence of large amounts of LAP in these samples. Although the data presented in Table I for the *S. typhimurium* (both filtrate and supernatant from filtrate) and the urine appear to indicate that no endotoxin was removed by passage through the columns, this is probably somewhat misleading. Clearly, the results shown in Fig. 3 (in which the fever was diminished) indicate that some of the endotoxin from the filtrate of *S. typhimurium* was removed by passage through the

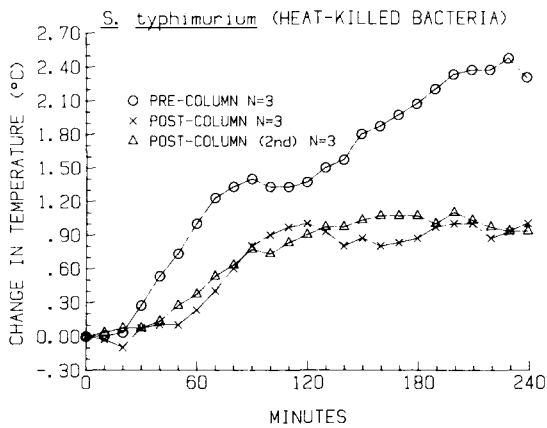

Fig. 3. Effect of injecting (1 ml, intravenously) pre- and post-polymyxin-Affi-Gel column solutions after repeated elution of filtrate of heat-killed *S. typhimurium* (LT-2 wild type from ATCC) on the mean (± 1 SE) change in rectal temperature of the New Zealand White rabbit. The pre-column solutions contained between 100 and 500 ng, based on a *Limulus* lysate assay.

TABLE I

REMOVAL OF ENDOTOXIN BY POLYMYXIN-AFFI-GEL COLUMNS, BASED ON *LIMULUS* LYSATE ASSAY

LPS from:	Concentration of endotoxin in pre-column sample (μg/ml)	Concentration of endotoxin in post-column sample (μg/ml)
E. coli (Sigma, 0111 : B4)	10	< 0.0005
E. coli (Difco, 0111 : B4)	10	0.1–0.2 [a]
E. coli (K-37)	> 0.5	0.0005–0.001
S. typhimurium (Sigma, L-6511)	10	< 0.0005
S. typhimurium (filtrate of bacteria)	0.1–0.5	0.1–0.5 [a]
S. typhimurium (supernatant from filtrate of bacteria	0.01–0.05	0.01–0.05 [a]
Urine	> 1	> 1: [a]
Urine (filtrate)	> 1	0.1–0.5 [a]

[a] Pyrogenic concentration (for reference purposes, the amount of endotoxin that will cause a fever in a 4-kg rabbit is i.v. injection of 1 ml of 0.004 μg endotoxin/ml).

column. It is possible that the failure to detect a reduction in endotoxin based on the *Limulus* lysate assay is attributable to the presence of particulate matter in these suspensions. In addition, since the values reported in the table represent ranges of endotoxin, it is possible that small amounts of endotoxin could be removed without detection by this assay.

No endotoxin was detected using the rabbit pyrogen test until 250 and 230 ml of 10 μg/ml *E. coli* (0111 : B4, Sigma) were run through columns at 22°C and 4°C, respectively. Since each column contained approximately 4 ml of gel, the total amount of *E. coli* endotoxin (Sigma) that can be removed using these columns is approximately 600 μg/ml of gel. In order to determine whether elution of higher *concentrations* of endotoxin would result in decreased binding, 10 ml of 100 μg/ml *E. coli* (0111 : B4, Sigma) were run through freshly prepared columns. No detectable pyrogen appeared in the eluent. We have not determined the maximum concentration of endotoxin that will bind to these columns.

These data indicate that polymyxin B bound to a gel support effectively inactivates some, but not all, endotoxins. As a result, we strongly urge that extreme caution be used before claiming a drug or immunologic agent endotoxin-free based on the use of polymyxin B.

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