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Relative Potencies of Four Reference Endotoxin Standards as Measured by the *Limulus* Amoebocyte Lysate and USP Rabbit Pyrogen Tests

MARLYS E. WEARY,* GEORGIANA DONOHUE, FREDERICK C. PEARSON, AND KENNETH STORY

Travenol Laboratories, Inc., Morton Grove, Illinois 60053

Four commonly used reference endotoxin standards, *Escherichia coli* O113:H10:K0, *E. coli* O55:B5, *Salmonella abortusequi*, and *Shigella dysenteriae* were compared by the USP rabbit pyrogen and the *Limulus* amoebocyte lysate tests. By the rabbit pyrogen test, *S. abortus equi* was identified as the most potent endotoxin, followed closely by *E. coli* O113:H10:K0 and *E. coli* O55:B5.

The sensitivity and relative simplicity of the *Limulus* amoebocyte lysate (LAL) test for the detection of bacterial endotoxin indicate that it can be used to replace the more elaborate, expensive, and variable rabbit pyrogen test prescribed in the *United States Pharmacopeia* (USP) (7) and in U.S. Public Health Service regulations for testing antibiotics (9) and biological products (8). However, an endotoxin standard must be used to compare the LAL test results generated by different laboratories, because differences in sensitivity of up to 100-fold have been found among different commercial LAL reagent preparations (10).

Four endotoxin standards were compared by using both the USP rabbit pyrogen and LAL tests to determine the relative potency of each. Sublots of *Escherichia coli* O113:H10:K0, a phenol Westphal-extracted preparation (6) designated EC-2 and adopted by the Bureau of Biologics (BuBio) of the Food and Drug Administration as a national endotoxin reference standard, were obtained from BuBio, Bethesda, Md. A commercial endotoxin, *E. coli* O55:B5, control 504089, a trichloracetic acid Boivan-extracted preparation selected by the Health Industry Manufacturer's Association as its reference standard for establishing equivalency of the LAL test to the USP rabbit test (4, 5), was obtained from Difco Laboratories, Detroit, Mich. A diethylene glycol-extracted preparation of *Shigella dysenteriae* (1), designated as the first international reference preparation for pyrogen by the World Health Organization (WHO) Expert Committee on Biological Standardization, was obtained from the National Institute for Biological Standards and Control, London, England. Novo Pyrexal, a phenol-extracted preparation of *Salmonella abortusequi* (3) (reextracted by phenol, chloroform, and petroleum ether; electrodialysis; and phenol, chloroform, and petroleum ether) was obtained from Hermal Chemie, Kurt Hermann, Hamburg, West Germany.

LAL reagents used were U.S. reference lot 4 (BuBio), Travenol lot 3338U003A (Travenol Laboratories, Inc., Morton Grove, Ill.), and Pyrogent lot 8EL (Mallinckrodt, St. Louis, Mo.).

Lyophilized endotoxins were reconstituted with sterile water for injection (USP) to a concentration of 0.1 μg/ml; graded doses of all endotoxins were then prepared in 0.9% NaCl for injection (USP) or sterile water for injection (USP) (Travenol Laboratories). All glassware used was depyrogenated by dry-heat treatment at 250°C or higher for at least 1 h.

Graded doses of each endotoxin preparation in normal saline were tested in rabbits once daily on each of 6 separate days by the three-rabbit test described in the *United States Pharmacopeia* (7). Endotoxin solutions were injected at 10 ml per kg of body weight into New Zealand White virgin does from the Travenol rabbit colony. The five doses prepared for each of the EC-2, *E. coli* O55:B5, and Novo Pyrexal endotoxins were 200, 100, 50, 25, and 12.5 pg/ml. Preliminary testing indicated that the WHO endotoxin was less pyrogenic, and the five test doses selected for it were 3,200, 1,600, 800, 400, and 200 pg/ml. A control solution, consisting of the same lot of normal saline that was used to prepare the endotoxin solutions each day, was also injected into three rabbits. The relative potency of an endotoxin as shown by the USP rabbit test was defined as that dose that produced a rise in temperature of 3.7°C ÷ 8 (>0.46°C) per rabbit.

LAL pyrogen tests were run on all four endotoxins on each of 3 days. Eight concentrations of each endotoxin in normal saline were prepared on each test day: 3,200, 1,600, 800, 400, 200, 100, 50, and 25 pg/ml. Four tests were run for each dose of each endotoxin. A negative control consisting of four samples of normal saline in com-

bination with each of the three LAL reagents was also prepared on each test day. The LAL gel clot test procedure involved combining 0.1 ml of endotoxin with 0.1 ml of LAL in test tubes (10 by 75 mm), incubating in a water bath at 37°C for 60 min, and then reading the tests by inverting the tubes. A test was positive if a solid gel clot was formed that remained at the bottom of the tube after inversion. A test was negative if the clot broke apart or if the contents of the tube remained liquid. Endpoint determinations were made by recording the smallest concentration of endotoxin that produced a solid gel clot. The LAL test procedure was repeated on all four endotoxins with sterile water for injection instead of normal saline used as the diluent. Only two of the three LAL reagents were available for this assay (BuBio and Travenol).

The natural logarithm of the endpoint was calculated for each test. An analysis of variance was run, using the natural logarithm of the endpoints as the dependent variable. The factors in the analysis of variance model are lysate, endotoxin, their interaction, and the date.

Dose-response curves for the four endotoxins are shown in Fig. 1. The daily mean dose concentration needed to produce a 0.46°C rise in temperature and the corresponding standard deviation of the estimate are listed in Table 1. Also listed are the mean dose concentrations needed to produce a rise across days and their standard deviations. The mean endotoxin doses (with their standard deviations within parentheses) producing a 0.46°C rise in temperature for Novo Pyrexal, EC-2, *E. coli* O55:B5, and WHO endotoxins were 56.59 (4.784), 93.51 (3.251), 121.42 (5.191), and 328.58 (156.521) pg/ml, respectively.

The concentration required for 0.46°C rise in temperature was determined from the 15 data points for each endotoxin-per-day combination. These 15 points consist of 3 data points at each of five concentrations. The dose-response curves in Fig. 1 and the concentration needed to produce a 0.46°C rise in temperature were calculated with the data from all 6 days. Table 2 contains the overall mean change in temperature for each endotoxin at each concentration and the standard deviations of the temperature changes.

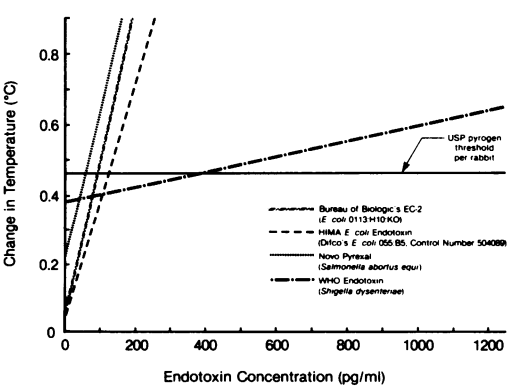

FIG. 1. *USP rabbit test dose-response curve for four endotoxins.*

According to these results, the endotoxins may be ranked as follows in decreasing order of potency: Novo Pyrexal, EC-2, *E. coli* O55:B5, and the WHO endotoxin. The first three are approximately three to six times as potent as the WHO endotoxin on the average. However, the WHO data show much more variability, as indicated by the standard deviations listed in Table 1.

Mean LAL gel clot endpoints expressed in picograms per milliliter for the four endotoxins

TABLE 1. *Concentration of endotoxin needed to produce a 0.46°C rise in temperature*

Endotoxin and day of dose	Endotoxin concn (pg/ml)	
	Mean	Standard deviation
Novo Pyrexal		
1	55.53	18.220
2	31.38	10.821
3	102.81	14.165
4	58.97	6.959
5	40.07	12.620
6	57.40	8.467
E. coli O55:B5		
1	93.87	7.419
2	144.90	9.175
3	93.76	7.453
4	111.63	10.106
5	105.85	9.834
6	356.71	122.354
EC-2		
1	91.02	9.053
2	93.74	6.400
3	105.21	7.962
4	91.59	5.939
5	89.43	9.323
6	91.92	7.458
WHO		
1	350.37	238.582
2	—[a]	—[a]
3	1008.71	229.004
4	−47.95[b]	474.854
5	260.63	363.620
6	−242.67[b]	674.735

[a] Data for this group for this day were not reported because they did not follow a normal distribution.

[b] Negative numbers reported here resulted from variations in the data so great that a negative slope was calculated for those days.

in normal saline and for the four endotoxins in sterile water that were tested with two lysates are shown in Table 3. The proportionate relationship of each endotoxin to the most potent endotoxin, Novo Pyrexal, is also shown. Statistical evaluation showed significant differences between test substances diluted with saline and those diluted with sterile water for lysate, endotoxin, and lysate-endotoxin interaction of $P < 0.0001$, < 0.0001, and $= 0.0015$, respectively. Because of the interaction, an analysis of variance was conducted on each lysate. Again, the natural logarithm of the endpoint was used as the dependent variable. The only factors in the analysis of variance were endotoxin and date.

TABLE 2. *Overall mean temperature change per rabbit and standard deviation*

Endotoxin concn (pg/ml)	Mean temp change (°C)	Standard deviation
EC-2		
0.0	0.05	0.092
12.5	0.12	0.091
25.0	0.17	0.122
50.0	0.24	0.214
100.0	0.55	0.214
200.0	0.89	0.255
Novo Pyrexal		
0.0	0.04	0.062
12.5	0.19	0.184
25.0	0.37	0.188
50.0	0.57	0.311
100.0	0.73	0.272
200.0	0.95	0.283
WHO		
0	0.04	0.062
200	0.44	0.490
400	0.59	0.226
800	0.61	0.360
1600	0.85	0.299
3200	0.96	0.192
E. coli O55:B5		
0.0	0.05	0.092
12.5	0.10	0.096
25.0	0.23	0.134
50.0	0.14	0.156
100.0	0.50	0.179
200.0	0.67	0.262

In each case there were significant endotoxin differences. A Duncan Multiple Range Test was performed at the $\alpha = 0.05$ level (2). The conclusion of the Duncan test was that for the BuBio and Travenol lysates, Novo Pyrexal was the most potent, followed by *E. coli* O55:B5, and that EC-2 and the WHO endotoxin were the least potent. For the Mallinckrodt lysate, Novo Pyrexal and *E. coli* O55:B5 were equally the most potent, and EC-2 and the WHO endotoxin were equally the least potent.

The above statistical tests were also performed on data for the endotoxins prepared in water. There were no lysate differences ($P = 0.69$), but because there was a significant lysate-endotoxin interaction ($P = 0.0012$), separate tests were run for each lysate. In each case there were significant endotoxin differences. A Duncan Multiple Range Test was performed at the $\alpha = 0.05$ level. Using the BuBio lysate, *E. coli* O55:B5 and Novo Pyrexal were equally the most potent, and EC-2 and the WHO endotoxins were equally the least potent. For the Travenol lysate, Novo Pyrexal, *E. coli* O55:B5, and EC-2 were equally the most potent, and WHO was the least potent.

In conclusion, (i) the WHO endotoxin is the least promising as an LAL test endotoxin standard because of its relatively low potency and

TABLE 3. *Comparative potency of four endotoxins determined by the USP rabbit and and LAL tests*[a]

Test	Comparative potency (pg/ml) for:			
	Novo Pyrexal	EC-2	*E. coli* O55:B5	WHO
USP rabbit pyrogen[b]	56.6 (1)	93.5 (1.7)	121.4 (2.1)	328.6 (5.8)
LAL[b]				
BuBio lysate	83.3 (1)	666.7 (8.0)	333.3 (4.0)	666.7 (8.0)
Travenol lysate	83.3 (1)	666.7 (8.0)	200.0 (2.4)	933.3 (11.2)
Mallinckrodt lysate	20.7 (1)	233.3 (11.2)	25.0 (1.2)	333.3 (16.1)
LAL[c]				
BuBio lysate	50.0 (1)	266.6 (5.3)	41.6 (0.8)	266.6 (5.3)
Travenol lysate	66.6 (1)	83.3 (1.25)	83.3 (1.25)	400.0 (6)

[a] The numbers in parentheses indicate the proportionate amount of endotoxin required to produce the same effect as the most potent endotoxin, Novo Pyrexal.
[b] Endotoxin diluted in normal saline.
[c] Endotoxin diluted in sterile water for injection.

its unusually high variability in the rabbit pyrogen test, (ii) EC-2 is a questionable choice as an LAL test endotoxin standard because it varies among lots and exhibits reduced LAL test activity in normal saline, and (iii) Novo Pyrexal and *E. coli* O55:B5 are the most appropriate selections for LAL test endotoxin standards, because their potencies with the LAL and USP rabbit tests are closely parallel and they exhibit little lot-to-lot variability.

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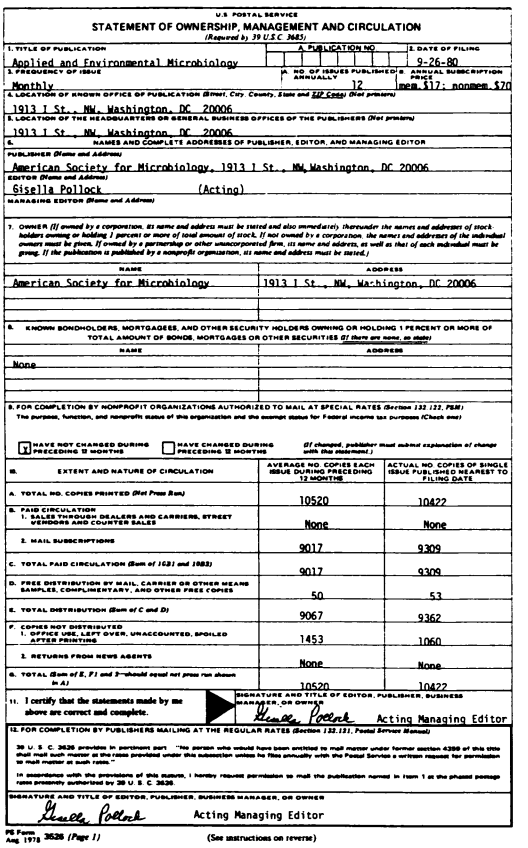

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