Preparation and Properties of a National Reference Endotoxin

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A large pool of refined endotoxin was prepared from *Escherichia coli* O113 by extraction with hot aqueous phenol. It was characterized chemically and biologically and will be available for a reference standard designated as reference endotoxin EC.

This report describes the preparation and properties of a refined bacterial endotoxin, which is being adopted as a national biological reference standard. Samples of this endotoxin will be available to pharmaceutical manufacturers and qualified biomedical investigators as an aid to standardization of bioassays and research with endotoxin. As an example, standardization would be helpful for the limulus amoebocyte lysate (LAL) test (4, 6, 12, 13, 23) and the standard test for pyrogenicity in rabbits (19).

The endotoxin described herein, extracted from *Escherichia coli*, is the second reference endotoxin. The first reference endotoxin, prepared from *Klebsiella pneumoniae*, was described previously (3, 18). However, the present report contains results of parallel assays with both the *E. coli* and *K. pneumoniae* reference endotoxins. The presentation of these results should facilitate the correlation of data based on the previous standard with those based on reference endotoxin EC.

MATERIALS AND METHODS

Cultivation and harvesting. The bacterial source of reference endotoxin EC has the following complete designation: *Escherichia coli* (Braude strain) O group 113:H10:K negative (5) (serotyping by Bacteriology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, Ga.). The culture was maintained on Trypticase soy agar slants. Before each use as an inoculum, it was checked for purity by streaking on a Trypticase soy agar plate, by Gram staining reaction, and by agglutinating reaction with specific antibody. All other cultures of this organism were grown in a medium (M-9) adapted (17) from the synthetic "ammonium medium" of Anderson (2). As employed here, it had the following composition per liter: NH_4Cl, 1 g;

Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 2 g; $MgSO_4$, 0.1 g; pH 7.2. Glucose, autoclaved separately, was added in the amount of 5 g per liter. For each batch of bacteria, three 500-ml flasks, each containing 100 ml of medium, were inoculated with *E. coli* O113. These flasks were incubated, with shaking, at 37 C for 18 h, and the cultures were again tested for purity as described above. The three cultures were inoculated into 14 liters of medium in the tank of a Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). The batch was grown for 18 h at 37 C with 400-rpm agitation and aeration of 3.5 liters/min at 8.5 lb/in². A 20% solution of Antifoam B (Sigma Chemical Co., St. Louis, Mo.) was added as needed. The bacteria were harvested in a Sharples continuous-flow centrifuge (Sharples-Stokes Division of Pennwalt Corp., Warminster, Pa.) and washed three times with cold phosphate-buffered saline (0.15 M NaCl; 0.0033 M PO_4; pH 7.2), and the wet weight was determined.

Extraction of endotoxin. The cells were suspended to a concentration of 10% in distilled water, and an equal volume of 88% liquid phenol (USP, J. T. Baker Chemical Co., Phillipsburg, N.J.) was added. The mixture was placed in a 65 C water bath and stirred until the temperature reached 65 C and the phenol and aqueous phases were homogenized. Stirring was continued for another 30 min, and the solution was distributed to glass centrifuge tubes, which were then chilled in an ice bath overnight to allow the phases to separate. The tubes were centrifuged at 4 C for 60 min at 1,000 × *g*. The aqueous phases, after removal from the inter- and phenol-phases, were combined. An equal volume of water was added to the combined phenol- and interphases, and the extraction was repeated; the second aqueous phase was combined with the first, and the phenol residue was discarded. The aqueous phases were dialyzed against running tap water at about 6 C for 7 days and against three daily changes of distilled water at 4 C. After dialysis, moisture was allowed to evaporate at 22 to 25 C through the dialysis bags until the combined aque-

ous phases were reduced to about $^1/_{10}$ of their original volume. This took 3 or 4 days. The above procedure was modified from that described by Westphal et al. (21).

Refinement and purification of reference endotoxin EC. The endotoxin was refined initially as follows (16). Sufficient sodium acetate was added to the aqueous phase to bring the concentration to 0.15 M; the solution was cooled, and 95% ethanol was added at 4 C, dropwise with stirring, to bring the alcohol concentration to 68%. After the suspension was left overnight at 4 C, the precipitate was collected by centrifugation at 1,000 × g for 30 min. The precipitate was dissolved in distilled water, dialyzed at 4 C against four daily changes of distilled water, and centrifuged at 1,000 × g for 30 min, and the supernatant fluid containing the endotoxin was frozen. Each batch of endotoxin was lyophilized separately; dry weights were determined, and preliminary chemical (nucleic acid, hexose, nitrogen) and biological analyses (LAL, pyrogen) were performed on each batch. Batches of the *E. coli* endotoxin with lower nucleic acid or nitrogen contents (from preliminary analysis) were each redissolved in 200 ml of distilled water. These individual batches were grouped according to similar nitrogen, nucleic acid, or hexose contents into four pools of about 2,200 ml each. The pH was adjusted to 5.0 with 1 N HCl, and 250 mg of ribonuclease (Sigma Chemical Co., 80 Kunitz units/mg) and 20 mg of deoxyribonuclease (Sigma Chemical Co., 587 Kunitz units/mg) were added to each pool. The pools were incubated overnight at room temperature (22 to 24 C) with continuous stirring. The pools were then dialyzed at 4 C against five daily changes of distilled water, and the contents of the dialysis bags were combined into two pools. One pool contained endotoxins with higher concentrations of nucleic acid, and one pool contained the endotoxins with lower amounts of nucleic acid. Additional nucleotides were removed from each pool by adsorption on Dowex 1–x4 (Cl⁻) resin (J. T. Baker Chemical Co., Phillipsburg, N.J.). To both pools, each containing about 3,000 ml with 5 mg of endotoxin per ml, 500-g amounts of washed resin were added, and the suspensions were stirred for 1 h at 25 C. The resin was removed from the endotoxin solutions by filtration through Whatman no. 1 filter paper. Preliminary chemical analyses of the solutions indicated that the nucleic acid had been reduced to less than 2.5% in both pools, so they were combined and freeze-dried.

Chemical analyses. Ketodeoxyoctulosonate and dideoxysugars were quantified by a modified thiobarbituric acid reaction as described previously (1). Heptoses were estimated by the method of Osborn (14). Nucleic acid in the endotoxin preparations was derived from ratios of absorbency readings at 260 and 280 nm, based on the relationship formulated by Warburg and Christian (20). The remaining analyses for nitrogen, phosphorous, hexose (anthrone), total carbohydrate (tryptophan-sulfuric acid), hexosamine (Elson-Morgan), and fatty acid ester plus fatty acid amide (expressed as palmitate) were determined by standard methods as described in detail previously (16).

Biological tests. Capacity of the endotoxins to cause gelation of lysate from the amoebocytes of *Limulus polyphemus* was estimated by established techniques (4). The LAL was prepared from amoebocytes lysed with distilled water as described previously (9). The test was performed with 0.1-ml quantities of varying dilutions of endotoxin added to 0.1 ml of limulus lysate, and the tubes were incubated at 37 C for 60 min. A positive reaction was indicated by the formation of a gel that remained firm when the tube was inverted.

The pyrogenic responses of rabbits to intravenously administered doses of endotoxin were determined both at the Laboratory of Clinical Investigation (22) and at the Rocky Mountain Laboratory (13) by standard procedures for determinations of either the minimum pyrogenic dose (MPD) (the dose expected to produce a rise of 0.6 C or 1.0 F) or the dose producing a fever index of 40 cm² (FI_{40}). The FI_{40} is a centering constant proposed by Keene et al. (10), and it was obtained in the following manner. Groups of rabbits were inoculated with graded doses of pyrogen within a predetermined range, and the temperatures were recorded at intervals for a period of 7 h after inoculation (22). The individual curves were plotted, with 1 C and 1 h each being allotted 1 inch (2.5 cm). For each animal, the area included between the temperature curve and the base line was obtained in square centimeters, by planimetry or by computer. This area was called the FI. The average FI, at each dose level, was then plotted against the logarithm of the dose, and the FI_{40}, or that dose expected to give an FI of 40 cm², was read from the graph.

The MPD of reference endotoxin EC was also estimated, because this value is more nearly related to the standard test for pyrogenicity (19), which all pharmaceuticals intended for intravenous injection must pass. This easier, but less satisfactory (10), method of quantifying the febrile response was based on the maximum rises in temperature, during 3 h of observation, after injection of graded doses of pyrogen. The MPD was read from a line fitted to the plot of average maximum rises against the logarithms of doses.

Lethality for chicken embryos was estimated as described previously (13). Embryos, after 11 days of incubation, were inoculated intravenously with doses contained in 0.1 ml. In each test, 10 embryos were inoculated with each of at least four graded doses, usually differing by fivefold. The results, after an additional 24 h of incubation, were used to calculate the median lethal dose for chicken embryos.

RESULTS

Over 77 g of endotoxin was extracted in 47 individual batches. After preliminary chemical and biological analyses were performed, the endotoxins from 32 batches (49 g) were considered superior and were pooled for further purification as described above; these additional steps were undertaken primarily to reduce the nucleic acid content of the endotoxin. After purification, 31 g of endotoxin (called reference endotoxin EC) was obtained in lyophilized

form. Thus, the overall yield of reference endotoxin EC was 40% of the total of the crude extracts. The final product, at concentrations of 2 mg/ml or less, went into almost clear solutions in water or saline, with little or no tendency to settle out on standing.

Samples of reference endotoxin EC were analyzed chemically and biologically at the University of Montana, the Rocky Mountain Laboratory, the Laboratory of Clinical Investigation, and the Bureau of Biologics. The results of gross chemical analyses obtained in different laboratories are given in Table 1. It can be seen that reference endotoxin EC has a chemical profile typical of refined endotoxins from enteric gram-negative bacteria (cf. reference 12). A low content of nucleic acids was specified to eliminate any possibility that the biological properties of the preparation could be ascribed to polynucleotides (4). Inasmuch as the O-somatic antigen of *E. coli* O113 does not contain colitose, the chemical analyses for dideoxysugars were negative.

A summary of the biological activities of reference endotoxin EC, as determined at the Rocky Mountain Laboratory, is presented in Table 2. Lethality for chicken embryos was assayed 21 times in the course of calibrating other preparations against this reference stand-

TABLE 1. *Gross chemical composition of reference endotoxin EC*

Constituent	Composition (%)
Nitrogen	2.60[a]
	2.66
	2.48
Phosphorus	1.35
Hexose	16.9
	27.6
	26.2
Total carbohydrate	34.4
Hexosamine	16.4
Fatty acid amide plus fatty acid ester	35.0
Ketodeoxyoctulosonate	3.88
Dideoxysugars	<0.1
Heptose	2.38
Nucleic acid	2.96
	2.20

[a] Where more than one value is listed, this indicates that each of the determinations was made in a separate laboratory.

TABLE 2. *Biological activities of reference endotoxin EC determined at the Rocky Mountain Laboratory*

Determinant	Biological activity (ng)
$CELD_{50}$[a]	
Mean	11.9
Range	5.8–29
SE	1.3
FI_{40}	
Assay 1	270
Assay 2	130
Assay 3	170
Mean	190
MPD	
Assay 1	0.46
Assay 2	1.4
Assay 3	0.71
Mean	0.86

[a] FI_{40}, Dose of endotoxin producing a fever index of 40 cm²; MPD, minimum pyrogenic dose, or dose producing rise in temperature of 0.6 C; $CELD_{50}$, mean lethal dose for 11-day-old chicken embryos inoculated intravenously; SE, standard error.

ard. The range of median lethal doses for chicken embryos was 5.8 to 29 ng; the mean, 11.9 ng; and the standard error of the mean, 1.3 ng.

Three determinations of FI_{40} (Table 2) gave values ranging from 130 to 270 ng; three of MPD ranged from 0.46 to 1.4 ng. The methods for these two types of quantitation are illustrated in Fig. 1. A somewhat higher value for the FI_{40} was obtained at the Laboratory of Clinical Investigation, where a parallel comparison of FI_{40} was also made between reference endotoxin EC and the earlier reference endotoxin from *K. pneumoniae* (Fig. 2).

In parallel tests of ability to clot LAL (Table 3), both of the reference endotoxins were shown to be active at the nanogram level.

DISCUSSION

The need for an available reference standard endotoxin has been appreciated by workers in the field for many years (11). Recently, the interest in the LAL test for the detection of endotoxin clinically and in pharmaceutical products has provided the impetus for preparation of reference endotoxin EC. Because different preparations of the lysate have shown extreme variations in sensitivity and because the tests are not read in the same way by all workers, meaningful comparisons can only be made in terms of potency of a given material relative to that of a standard.

Reference endotoxin EC meets most, if not all, of the general requirements for an inter-

national standard (7). It is a dry preparation available in substantial quantity. Endotoxins of this kind are known to be remarkably stable at room temperature and, being only slightly hygroscopic, they may be stored without special protection against atmospheric moisture. Steps are being taken to make the standard readily available. In addition, reference endotoxin EC has a chemical composition and biological potency within the limits of the requirements established by the Bureau of Biologics (8). It is referred to as "soluble" in a broad sense, indicating that it goes readily into an apparently

colloidal aqueous solution, clear except for a faint opalescence, and has (thus far) no observable tendency to settle out on standing.

Minor discrepancies between results obtained in different laboratories were no greater than should be expected. Determinations of pyrogenicity, in particular, are subject to considerable variability, not only among different stocks and ages of rabbits (as illustrated by results in two laboratories, reported here) but also among repeated tests under the same conditions (Table 2). All biological tests classed the new standard as a highly potent endotoxin.

As described above, reference endotoxin EC consists of a single batch of powder dried
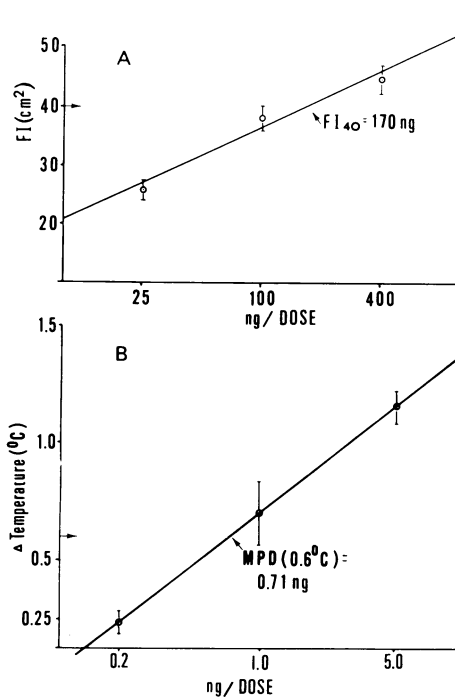

FIG. 1. *Pyrogenicity of reference endotoxin EC as determined at the Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases. (A) Graphical estimation of FI_{40} from the average FI's of 24 rabbits per dose level. (B) Example of the graphical estimation of MPD. Responses of eight rabbits per dose level are represented. Vertical bar indicates range of 1 standard error of each mean.*
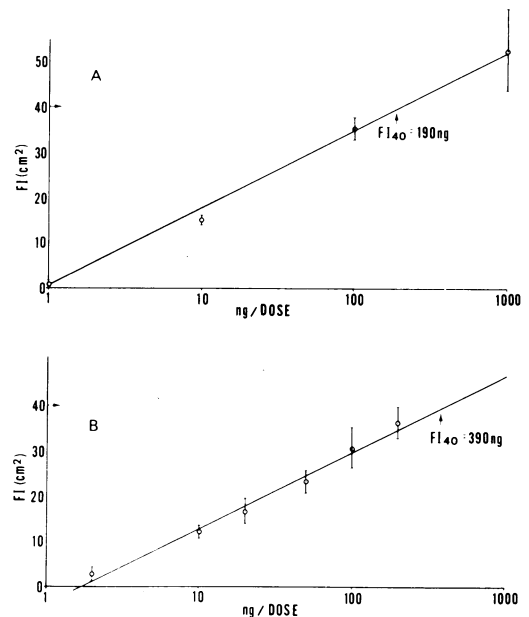

FIG. 2. *Pyrogenicity of reference endotoxins as determined at the Laboratory of Clinical Investigation. (A) Estimation of FI_{40} of reference endotoxin from K. pneumoniae. Three or four rabbits were used for each dose level. (B) Estimation of FI_{40} of reference endotoxin EC from E. coli O113; six to nine rabbits were used per dose level. Vertical bar indicates range of 1 standard error of each mean.*

TABLF 3. *Results of limulus lysate analysis[a]*

Endotoxin	Endotoxin concn (ng/ml)									
	100	50	12	10	6	5	3	1	0.5	Control
Reference endotoxin EC	+[b]	+	+	+	+	+	+	+	−	−
Klebsiella standard	+	+	+	+	+	−	−	−	−	−

[a] These tests were performed, with the same batch of lysate, six times in two laboratories. Replicate assays averaged less than one tube variation from the representative end points presented.

[b] +, Gel remains firm when the tube is inverted.

from a large pool. For distribution, it is proposed that samples of this primary batch be removed, dissolved at suitable concentration with 0.1% human serum albumin as a filler, dispensed into a quantity of vials, and freeze-dried. Analyses in the Laboratory of Clinical Investigation and the Bureau of Biologics (unpublished data) confirmed that lots of freeze-dried reference endotoxin EC prepared in this manner retain their full potency after reconstitution, as determined by rabbit pyrogenicity and LAL reactivity. These lots will be designated by consecutive numbers. The first will be reference endotoxin EC lot no. 1. Each lot will be, again, tested biologically before distribution. Information concerning the availability of reference endotoxin EC for standardization of biological assays may be obtained from the Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Rockville, Md. 20014.

ACKNOWLEDGMENT

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