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Comparison of Several Control Standard Endotoxins to the National Reference Standard Endotoxin—an HIMA Collaborative Study†

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A collaborative study, initiated under the auspices of the Health Industry Manufacturers Association (HIMA), was designed to establish the relationship of *Escherichia coli* O55:B5 endotoxin (the control standard endotoxin of HIMA and the Food and Drug Administration's Office of Medical Devices) to the U.S. National Reference Standard Endotoxin and to two internationally used control standard endotoxins. By using two *Limulus* amoebocyte lysate test systems, it was established that the *E. coli* O55:B5 endotoxin lot originally used by HIMA and the Office of Medical Devices to establish *Limulus* amoebocyte lysate release test criteria for pyrogen testing of medical devices contains approximately 4.5 endotoxin units (EU) per ng. Thus, the 1.0-ng/kg endotoxin dose limit currently established for medical devices is approximately the same as the 5.0-EU/kg endotoxin limit (on an activity basis) established by several other Food and Drug Administration agencies for human and animal parenteral drugs and biological products.

This study was carried out to develop conversion factors to relate the potency of three control standard endotoxins (CSEs) to that of the current National Reference Standard Endotoxin (RSE) *Escherichia coli* O113:H10:K0, which is identified by the U.S. Pharmacopoeia (USP) as lot F and by the U.S. Food and Drug Administration (FDA) as lot EC-5. The study was sponsored by the Health Industry Manufacturers Association (HIMA) and was encouraged by the FDA's Office of Medical Devices (OMD) as an effort to relate the potency of their joint CSE, Difco Laboratories' (Detroit, Mich.) *E. coli* O55:B5, to that of the RSE in endotoxin units (EU). The selection of *E. coli* O55:B5 as the official CSE of the OMD was the result of a previous HIMA collaborative study (1) that established this endotoxin as a CSE for the *Limulus* amoebocyte lysate (LAL) assay for medical devices by characterizing its potency utilizing the USP Pyrogen Test (9). That study concluded (at a 95% confidence interval) that an average rabbit pyrogen test laboratory would attain a 50% pass/fail rate with *E. coli* O55:B5 endotoxin concentrations above 98 pg/ml (or ca. 1.0 ng/kg when the endotoxin is administered at 10 ml/kg per the USP pyrogen test). Therefore, the HIMA task force recommended that 0.1 ng/ml of *E. coli* O55:B5 endotoxin be the pass/fail limit against which LAL pyrogen test results of medical devices be compared. Subsequently, this recommendation was accepted by the OMD and appears in their draft guidelines for the use of the LAL test, first issued on March 26, 1979. On March 29, 1983, the FDA announced the availability of a single multiagency draft guideline setting forth procedures for the validation of the LAL test as an end product endotoxin test for human and animal parenteral drugs, biological products, and medical devices (8). This document explained that although the FDA was issuing a single guideline, there were slight differences in the requirements for different classes of products because of variations in operational procedures among the FDA agencies that compiled the joint guideline. Among those differences was their selection of endotoxin standards. OMD retained its historical CSE, *E. coli* O55:B5, and all other FDA agencies selected the USP's RSE, which is identical to the USP standard lot EC-5, because both were prepared simultaneously from the USP's RSE.

The history and potency of the U.S. RSE, *E. coli* O113:H10:K0, has been widely reported (3, 6, 7). One vial of the current U.S. standard lot EC-5 contains 10,000 EU. The FDA guidelines provide a method for characterizing control standard endotoxins in terms of the U.S. standard. This study uses a similar procedure to establish the relationship of three well-known control endotoxin standards to the U.S. and USP reference endotoxin. The three control standard endotoxins are as follows: (i) Difco's *E. coli* O55:B5; (ii) the World Health Organization (WHO) standard prepared from *Shigella dysenteria* (2); and (iii) Novo Pyrexal, a *Salmonella abortus equi* endotoxin (4).

The HIMA collaborative study was conducted in two phases. Phase I consisted of an evaluation of the test protocol by five licensed manufacturers of LAL and was initiated to assess the within-laboratory variability that could be anticipated in a wider study. Phase II, carried out by eight LAL laboratories from industry, government, and academia,

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TABLE 1. Phase I study: geometric mean potencies of three control standard endotoxins relative to RSE EC-5

Lysate	Endotoxin	Geometric mean relative potency (EU/ng)	95% confidence interval for geometric mean relative potency (EU/ng)
Pyrogent	O55:B5[a]	4.13	(2.18, 7.83)
	WHO	3.32	(2.28, 4.83)
	Novo	5.99	(3.28, 10.92)
Pyrotell	O55:B5	5.22	(2.71, 10.09)
	WHO	1.05	(0.26, 4.18)
	Novo	6.43	(2.76, 14.96)

[a] Lot no. 019721A.

TABLE 2. Phase II study: geometric mean potencies of three control standard endotoxins relative to the RSE EC-5

Lysate	Endotoxin	Geometric mean relative potency (EU/ng)	95% confidence interval for geometric mean relative potency (EU/ng)
Pyrogent	O55:B5[a]	4.26	(3.23, 5.62)
	WHO	2.49	(1.75, 3.56)
	Novo	5.15	(3.70, 7.18)
Pyrotell	O55:B5	4.74	(3.92, 5.73)
	WHO	0.50	(0.33, 0.78)
	Novo	4.73	(3.03, 7.38)

[a] Lot no. 504089.

was, with one exception, identical to the protocol used in phase I. In phase I, the Difco *E. coli* O55:B5 endotoxin was identified as lot 019721A. Because the *E. coli* O55:B5 lot was not the same lot used in the 1978 HIMA rabbit pyrogen test study, a direct comparison could not be made between the two collaborative efforts. Therefore, the phase II study corrected this situation by replacing Difco lot 019721A with vials of endotoxin from Difco lot 504089, the same endotoxin lot used in the 1978 HIMA collaborative rabbit pyrogen test study.

MATERIALS AND METHODS

The following reagents were used for the protocol study: (i) U.S. Standard Endotoxin lot EC-5 (10,000 EU/vial); (ii) *E. coli* O55:B5 endotoxin (2.5 mg per vial; lot 019721A for the phase I study and lot 504089 for the phase II study; Difco); (iii) WHO *Shigella dysenteriae* endotoxin (1.0 μg per vial; lot 79/502); (iv) Novo Pyrexal (*S. abortus equi* endotoxin; 0.1 g/ml per vial; lot no. 730-3; Forte, Hermal Chemie, Kurt Hermann, Reinbek B. Hamburg, Federal Republic of Germany); (v) LAL (a) Associates of Cape Cod Pyrotell; lot 96-17-275, labeled sensitivity of 0.06 EU/ml against EC-5; (b) Mallinckrodt Inc., Pyrogent, lot 2FY, labeled sensitivity of 0.06 EU/ml against EC-5; (vi) sterile water for injection or irrigation, USP.

All glassware used in the study was depyrogenated for a minimum of 3 h at a minimum of 180°C or for 1 h at 250°C.

All testing at each laboratory was performed by a single technician on each of 2 days. On each day the technician evaluated the four endotoxins by using two LAL gel clot systems (Pyrogent and Pyrotell). Four replicate assays were performed each day for each endotoxin-lysate combination. Each replicate consisted of a set of twofold serial dilutions of the endotoxin ranging from 400 to 1.5 pg/ml or, in the case of EC-5, from 4 to 0.015 EU/ml accompanied in each case by a negative control. The endpoint assay value for each replicate set was the lowest endotoxin concentration at which the lysate formed a solid gel clot. The log 10 of each endpoint assay value was determined, and the values for each quadruplicate series were averaged to calculate the geometric mean endpoint for each endotoxin. The geometric mean endpoint was expressed in EU per milliliter for EC-5 and in picograms per milliliter for the other three endotoxins (9).

For purposes of statistical evaluation, the difference on a logarithmic scale between the mean for each control standard endotoxin and the mean for EC-5 was computed for each laboratory-date-lysate combination. For each control standard endotoxin, the mean difference was then computed across all laboratory-date combinations, and a variance component analysis was performed on the differences. By using the means and variance components, 95% confidence intervals were computed on the logarithmic scale. Conversion of logarithms back to the arithmetic scale yielded the geometric means and associated confidence intervals shown in Tables 1 and 2.

The variance component analysis used the difference on the logarithmic scale as the dependent variable and included an effect for laboratory (5). Day-to-day differences within a laboratory constituted the error term in the model. The 95% confidence intervals were computed with the appropriately weighted sum of the two variance components described above.

RESULTS AND DISCUSSION

Results for the phase I study are shown in Table 1, and results for the phase II study are shown in Table 2. The geometric mean potencies expressed in EU per nanogram for each of the three control standard endotoxins are remarkably similar for both data sets. In each case, the endotoxin geometric mean potencies produced during the phase II study fall within the 95% confidence intervals established by the phase I study.

In general, the intervals for both studies approach those of the twofold dilution limits considered to be statistically acceptable for the gel clot endpoint LAL assay. *E. coli* O55:B5 and the Novo Pyrexal endotoxin produced relative geometric mean potencies that were insignificantly different as measured by the two lysate test systems. However, the WHO endotoxin produced consistently higher relative potency with Pyrogent than with Pyrotell, reinforcing the requirement of the FDA guidelines and the USP Bacterial Endotoxins Test (9) that the CSE must be recalibrated to the RSE whenever the LAL used is changed. The WHO endotoxin was the weakest of the three control standard endotoxins, and in the Pyrotell lysate test system, it failed to meet the USP Bacterial Endotoxin Test requirement that a CSE possess a potency of not less than 2.0 EU/ng. Finally, it can be established that the Difco *E. coli* endotoxin lot 504089 used for the original HIMA rabbit pyrogen test collaborative study to set the endotoxin test limits used today by OMD to evaluate pyrogen test results of medical devices contains approximately 4.5 EU/ng when compared with the RSE with Pyrogent and Pyrotell lysates. Thus, the pass/fail endotoxin dose limit (on an activity basis) for medical devices of 0.1 ng/ml (or 1.0 ng/kg when injected into rabbits at 10 ml/kg) is approximately the same as the 5.0-EU/kg endotoxin dose limit established for drugs and

biological products in the FDA's draft guideline for the validation of the LAL test.

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