APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1985, p. 1509–1511
0099-2240/85/121509-03$02.00/0

Vol. 50, No. 6

Studies on the Sensitivity and Specificity of the *Limulus* Amebocyte Lysate Test and Rabbit Pyrogen Assays

M. J. DEVLEESCHOUWER,* M.-F. CORNIL, AND J. DONY

Service de Microbiologie et d'Hygiène, Institut de Pharmacie, Université Libre de Bruxelles, Campus de la Plaine, CP205/2, 1050 Brussels, Belgium

Received 19 February 1985/Accepted 10 August 1985

The sensitivity and specificity of the *Limulus* amebocyte lysate test and rabbit pyrogen assay were studied by means of artificially contaminated parenterals. Various gram-negative and gram-positive bacterial strains were used as was one strain of the yeast *Candida albicans*. The numbers of organisms needed to elicit positive responses in distilled water and normal saline were recorded and compared. The sensitivity and specificity of the *Limulus* amebocyte lysate assay for the detection of bacterial endotoxin from gram-negative bacteria were demonstrated. Variable results were recorded with gram-positive bacteria and *Candida albicans*.

Numerous studies have showed that the *Limulus* amebocyte lysate (LAL) test is a viable alternative to the compendious rabbit test for pyrogenicity in that the former is more sensitive, rapid, and inexpensive (12, 18). However, the vast majority of these studies have focused on gram-negative endotoxin. Although gram-negative endotoxin is the principal cause of pyrogenicity in parenteral solutions and medical devices (9, 18), gram-positive bacteria, mycobacteria, fungi, and viruses may also elicit a pyrogenic response; furthermore, the levels at which these organisms produce a pyrogenic response varies considerably (3, 7, 8, 13, 14, 16, 17, 20, 21). Thus, in the following study, detection of pyrogenicity by both LAL and the pharmacopeial rabbit test (2) are compared by using a number of organisms other than gram-negative bacteria. In addition, the correlation between certain well-defined sterilization conditions and the number of gram-negative organisms needed to elicit a positive response is examined.

MATERIALS AND METHODS

Sterile water and 0.9% sodium chloride solution (Travenol Laboratories, Lessines, Belgium) for injection were inoculated with between 10 and 10^7 organisms per ml in 1-log increments, allowing comparison of the number of organisms needed for each test to yield a positive response.

Test organisms were obtained from the American Type Culture Collection (ATCC) and were chosen either with respect to their known pyrogenicity, in the case of gram-negative rods of the family *Enterobacteriaceae* and *Pseudomonas* species, or to determine the test responsiveness in the case of gram-positive cocci or yeasts. The strains used were *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas putida* ATCC 12633, *Serratia marcescens* ATCC 13880, *Escherichia coli* ATCC 11229, *Klebsiella pneumoniae* ATCC 10031, *Staphylococcus aureus* ATCC 8095, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus pyogenes* ATCC 14289, and *Candida albicans* ATCC 10211. Cultures were made in appropriate fluid medium. The first seven organisms listed were incubated overnight in 30 ml of tryptic soy broth at 30 to 35°C. For *Streptococcus pyogenes*, incubation was similar, except Todd-Hewitt broth was used. *Candida albicans* was incubated at the same temperature for 2 days in Sabouraud dextrose broth.

After incubation, the strains were centrifuged, washed five times with sterile and apyrogenic normal saline, and finally suspended in about 30 ml of sterile water for injection. Cells were counted with a Tomà cell, and dilutions were made in the solution to be tested.

The various bottles were then inoculated and homogenized. A plate count was made immediately after dilution with peptone buffer and plating samples on tryptic soy agar (strains 1 to 7), blood agar (strain 8), or Sabouraud dextrose agar (strain 9). Plates were incubated for between 2 and 5 days, depending on the strain; after counting, the number of organisms was expressed in milliliters of solution. All of the bottles were subsequently autoclaved at 113.5°C for 40 min. After this treatment, either a rabbit pyrogen test or an LAL test was performed on samples from the various containers. The rabbit pyrogenicity test was performed in accordance with European pharmacopeial prescriptions (2); three rabbits per sample were inoculated at a dose of 10 ml/kg of body weight. The test consists of measuring the rise in body temperature evoked in the rabbits by intravenous injection of the solution to be examined.

For the LAL assay, the method previously published by Nandan and Brown (10) was used. For this method, quantities of *Escherichia coli* O55:B5 endotoxin (5.0 endotoxin units [EU]/ng) are used to prepare standards in 0.9% saline and distilled water. The amount of endotoxin-precipitable protein is quantitively determined for both standards and samples. Indeed, when increasing concentrations of *E. coli* endotoxin are added to a constant amount of LAL and the reaction is allowed to proceed to completion, there is a proportional increase in the protein precipitated by endotoxin.

Therefore, in measuring the amount of protein precipitated from LAL by the Lowry method for total protein, it is possible to determine the equivalent *E. coli* endotoxin concentration in unknown solutions (10). The results are expressed as picogram equivalents of *E. coli* O55:B5 endotoxin per milliliter.

The rabbit assay was interpreted in accordance with previously published material (2) as follows. The material is considered nonpyrogenic when the summed responses do not exceed 1.15°C. The material is pyrogenic when the summed responses exceed 2.65°C. For responses between 1.15 and 2.65°C, a new trial should be performed with three different rabbits. For the LAL test, a value equivalent to

* Corresponding author.

TABLE 1. Comparison of organism contents[a] needed to elicit a pyrogenic response in the LAL and European pharmacopoeial three-rabbit assays

Organisms	No. of organisms/ml by the following sterilization procedures			
	Saline		Water	
	LAL[c]	Rabbit[d]	LAL	Rabbit
Pseudomonas aeruginosa ATCC 15442	1.3×10^5	2×10^6	10^5	10^5
Pseudomonas putida ATCC 12633	1.8×10^4	2.9×10^5	3×10^3	1.6×10^6
Serratia marcescens ATCC 13880	8.9×10^4	10^6	10^5	10^6
Escherichia coli ATCC 11229	1.1×10^5	1.1×10^5	3.6×10^5	3.6×10^5
Klebsiella pneumoniae ATCC 10031	3.6×10^4	5×10^5	2.2×10^4	2×10^5
Staphylococcus aureus ATCC 8095	9.8×10^6	[b]	1.4×10^6	[b]
Staphylococcus epidermidis ATCC 14990	[b]	[b]	[b]	[b]
Streptococus pyogenes ATCC 14289	[b]	3.1×10^6	[b]	8×10^5
Candida albicans ATCC 10231	5.5×10^6	5.5×10^6	5.6×10^5	1.9×10^6

[a] Samples were inoculated with between 10 and 10^7 organisms per ml in 1-log increments.
[b] Positive response was not elicited at highest number tested.
[c] With the LAL test, all samples exceeding the value of the 50-pg equivalent of *E. coli* O55:B5 endotoxin per ml were considered positive (0.25 EU/ml).
[d] For the European pharmacopoeial three-rabbit assay, the material is considered pyrogenic when the summed rise of temperature exceeds 2.65°C.

0.05 ng or 50 pg of *E. coli* 055:B5 endotoxin per ml was taken as the pass-fail dose criterion for the test procedure. All samples exceeding this value were considered LAL positive.

RESULTS

The number of organisms per milliliter needed to produce an LAL-positive response and a rabbit pyrogenic response are summarized in Table 1. For *Pseudomonas aeruginosa*, LAL was positive at 10^5 organisms per ml. Rabbit pyrogenic responses occur at the same level or at 1 log higher. For *Pseudomonas putida*, an LAL-positive reaction was noted between 10^3 and 10^4 organism per ml; the rabbit test pyrogenic response was positive at 10^5 organism per ml or more, that is, at about 1 to 3 logs more.

For members of the family *Enterobacteriaceae*, especially *Serratia marcescens*, about 10^5 organisms per ml were necessary to obtain a response exceeding 50 pg of endotoxin per ml with the LAL test, whereas a rabbit pyrogenic response was obtained with a 1-log increase in organisms. With *E. coli*, 10^5 cells per ml elicited a positive response with the two methods, whereas for *Klebsiella pneumoniae*, a positive LAL reaction was elicited with about 10^4 bacteria per ml; rabbits gave a pyrogenic response with a 1-log increase. When considering the cocci, for *Staphylococcus aureus* under the conditions tested, about 10^5 organisms per ml were necessary to exceed 50 pg of endotoxin per ml with the LAL assay, whereas no pyrogenic response was recorded with the rabbits. For *Staphylococcus epidermidis*, no LAL-positive response was recorded with bacterial counts as high as 10^7 per ml. On the other hand, *Streptococcus pyogenes* elicited a pyrogenic response with rabbits at a concentration of 10^6 cells per ml and no LAL-positive reaction. Finally, for the yeast *Candida albicans*, 10^5 to 10^6 organisms per ml were necessary for an LAL-positive response, and the rabbit pyrogenic response occurred with 10^6 organisms per ml.

DISCUSSION

For the gram-negative organisms *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Serratia marcescens*, and *Klebsiella pneumoniae*, it is clear that the LAL test requires lower amounts of bacteria to be considered positive but that relatively high numbers of organisms are needed to elicit positive responses in both tests. The *E. coli* positive responses in the two tests are achieved at the same bacterial level. It is interesting to note that the USP chose an *E. coli* endotoxin as reference standard endotoxin (11). In addition, the concentrations needed to yield a positive response with the rabbit assay are in a much closer range (10^5 to 10^6 cells per ml for all organisms) than they are for LAL (10^3 to 10^6 cells per ml). Several other variations in responsiveness can be emphasized. Differences in assays are recorded among strains in the same solution.

When considering the same strain, differences are also observed when comparing the results with normal saline or distilled water. These variations in responsiveness for different strains or for the same strain in several tested products are also reported in the literature (8, 16, 17, 19).

When using gram-negative organisms in the first part of our study, the aim was to correlate some well-defined sterilization conditions (113.5°C for 40 min) with the number of gram-negative bacteria necessary to obtain positive responses with the two assays. Literature results on this subject are contradictory. For example, Dolder and Limacher (1) did not record a positive response for either test with counts of *Pseudomonas picketti* higher than 10^6/ml, and positive reactions were recorded only after 2 or 3 months. On the other hand, Jorgensen and Smith (5) obtained positive LAL reactions with whole *Pseudomonas aeruginosa* cells with contaminated infusion fluids. They stated that measurement of bound or whole endotoxin may be employed as a method of obtaining an approximate quantitation of gram-negative bacilli per volume of a particular fluid (6). In our opinion, correlations can be made between the bacterial counts and endotoxin content, but under well-defined conditions. Correlations made after sterilization will probably give results that are valid only under the stated conditions.

The second goal of our study was to determine whether gram-positive cocci or yeasts could elicit a positive response in either the LAL or rabbit test. Jorgensen and Smith (4) did not observe endotoxin-like activity with 11 different strains of yeasts tested with LAL, but this work was done with living cultures and not after a sterilization process. For the yeasts *Candida albicans*, we obtained responses for both trials with about 10^6 cells per ml. However, based on our test system, we cannot be sure that all of the endotoxin from the growth medium was washed off. Endotoxin is a common contaminant of microbial growth media and has been reported to cling tenaciously to yeasts (13).

As early as 1945, studies performed with rabbits (14) showed that pyrexia could be induced by gram-positive

bacilli and gram-positive cocci. These authors also found that the pyrogenicity of these organisms was reduced by autoclaving. If we consider more recent papers dealing with the LAL test, Reinhold and Fine (15) did not obtain gelation with log-phase cultures (10^9 cells per ml) of *Pneumococcus* sp., *Staphylococcus aureus*, and *Streptococcus hemolyticus*. The same observations were made by Wildfeuer et al. (20, 21) with intact cells of *Streptococcus pyogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Micrococcus lysodeikticus*. However, these authors and others (3, 7) give evidence that although peptidoglycans, lipoteichoic acids, and cell wall digests of gram-positive cocci can promote the gelation of LAL, the substances are less potent than control endotoxins used in these studies.

In our experiments with gram-positive cocci, the only positive response recorded with LAL was for *Staphylococcus aureus* (10^6 cells per ml). A rabbit pyrogenic response was only recorded with *Streptococcus pyogenes* at about 10^6 cells per ml. An explanation for the absence of reactivity in the other cases could be either insufficient destruction of the cells during autoclaving or a quantity of organisms too low to elicit a response.

Based on our results, we can conclude that the LAL assay is a sensitive and specific assay for the detection of bacterial endotoxin from gram-negative bacteria which can infrequently contaminate infusion fluid. A comparison of the pyrogenicity assays also indicates that the LAL is both more sensitive and more accurate.

The authors gratefully acknowledge the assistance of Travenol International Services, Lessines, Belgium.

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