

## Letter From the President



Dear LAL User:

*The possible presence of non-endotoxin pyrogens in injectable drugs, biologicals, and devices remains a concern of regulators and pharmaceutical manufacturers regardless of the successful track record of LAL and the added safety afforded by cGMP in controlling microbial contamination. An in vitro test which complements the LAL assay with the goal of completely eliminating an animal test is a highly commendable goal. However, unless the nature of endotoxin contamination of putative microbial and non-microbial non-endotoxin pyrogens is completely understood and controlled, an in-vitro test will only add confusion and cost to drug development while making no contribution to health and safety. This UPDATE is an introduction to some misconceptions regarding non-endotoxin pyrogens. Hopefully, with complete understanding, a useful in vitro pyrogen test can be realized.*

Sincerely,

Thomas J. Novitsky, Ph.D.

## BET vs. PT Non-Endotoxin Pyrogens

by Thomas J. Novitsky, Ph.D.

Since 1977 the US Food and Drug Administration (FDA) has accepted the *Limulus* amoebocyte lysate (LAL) test as an alternative to the United States Pharmacopeia (USP) Pyrogen Test <151> (PT).<sup>1,2</sup> Acceptance was based on a number of studies showing excellent correlation of the LAL assay with the PT.<sup>3,4,5</sup> Fred Pearson and colleagues summed this up nicely with the statement: "While several hundred thousand LAL tests have been conducted by Travenol, and several hundred have required retest in rabbits, in no case has there been an LAL pass and a rabbit failure."<sup>5</sup> As an added benefit, the LAL assay was found to be more sensitive than the PT, allowing realistic in-process control during the manufacture of drug solutions. Some studies examining the utility of LAL continued even after approval of the LAL test by the FDA. One study in particular showed that: 1) most drugs produced in the US were LAL negative, i.e. had no detectable endotoxin (2449 out of 2526, or 97%); 2) those that were positive were mostly drug products derived from natural sources (17 out of 24); and 3) endotoxin was associated with the contamination of raw material(s), not the finished product.<sup>6</sup> This did not come as too much of a surprise as even the earliest studies, used to validate the use of the febrile response in rabbits as the forerunner of the pharmacopeial PT, implicated endotoxin as the cause of "injection fever".<sup>7,8</sup> Thus, experimental and historical data overwhelmingly supported replacement of the PT with the BET. In the final analysis, the most serious objection to replacing the PT with the BET was not the issue of non-endotoxin pyrogens but the fact that the LAL assay was more sensitive than the PT, and the LAL activity with different endotoxins did not tightly correlate with PT response, i.e. the same weight of endotoxin from different bacteria did not all exhibit similar responses in the PT. Thus, the LAL test was not considered a good indicator of "biological activity". The first criticism was removed following studies which determined the actual amount of standard endotoxin needed to elicit a positive PT. This finding resulted in the concept of the "endotoxin limit". The second objection was also overcome with the introduction of the Reference Standard Endotoxin (RSE), and an accompanying unit of activity--the Endotoxin Unit (EU). So successful was the use of LAL and accompanying RSE that, beginning in 1985 with USP XXI, the USP began replacing the PT with the Bacterial Endotoxins Test (BET) <85> in monographs requiring a test for pyrogens. Water for Injection, recognized as a critical ingredient in nearly all injectables, or as a processing reagent, was the first monograph requiring the BET. In USP XXIV, only 20 monographs still specified the PT, all the rest required BET. On the other hand, the FDA, recognizing the possibility that a new drug may possess some inherent pyrogenicity (i.e. non-endotoxin pyrogen) or whose process may introduce a non-endotoxin pyrogen, still requires the PT as part of an Investigatory New Drug (IND) application for injectables.



Although this historical account of the replacement of the PT with the BET sounds reasonable and straightforward, those of us closely involved with LAL during this time remember the lively discussions and controversy accompanying LAL's bid for acceptance. No discussion ever escaped at least some mention of non-endotoxin pyrogens. At the crux of this matter was the fact that while the majority of endotoxins were pyrogens, several recognized pyrogens were not endotoxins. In his book, "Pyrogens. Endotoxins, LAL Testing, and Depyrogenation", Frederick C. Pearson III, presents an excellent overview of non-endotoxin pyrogens.<sup>9</sup> Although somewhat dated, his list is comprehensive. Classes of non-endotoxin pyrogens include microbial pyrogens (gram positive bacteria and some of their components, viruses and some of their components, and fungi and some of their components); and non-microbial pyrogens including certain antigens, steroids, polynucleotides, and synthetic adjuvants. It should be noted that even the most potent non-endotoxin pyrogens (with the possible exception of fungal glucan, see box) are orders of magnitude less potent than endotoxin. For example, while staphylococcal enterotoxin is considered a "potent" pyrogen, a concentration of 1 ug/kg is required to elicit a pyrogenic response in the PT. When compared to endotoxin (threshold pyrogenic dose of 0.5 EU (~ 0.05 ng/kg), the difference is 20,000 fold! Thus the likelihood of sufficient staphylococci contaminating a drug produced under cGMP conditions to elicit a pyrogenic response is remote. Likewise, many microbial components once thought to be pyrogens have since been shown to be contaminated with endotoxin. A recent example is lipoteichoic acid (LTA), which was recently shown to lose its ability to stimulate macrophages once adequately purified (i.e. LPS removed).<sup>10</sup> In an even more recent paper, Morath et al concluded: "Taken together, these data indicate that these crude preparations [of lipoteichoic acid] with relatively high endotoxin contamination are not suitable for characterizing the activation of immune cells by LTA."<sup>11</sup> Many "pyrogens" referenced in the early literature most likely fall into this category. Since endotoxin is ubiquitous in nature, and because it is "sticky", endotoxin is likely to be found in almost all products of biological origin even though it may be difficult to detect chemically. Most gram-positive organisms, for example, are grown in media (e.g. nutrient broth) that contain massive amounts of endotoxin. Thus, endotoxin arising from the media can stick to the cells and their components and remain as a contaminant unless extreme measures are taken to insure its removal.

In any case, with the acceptance by the USP, EP, JP, and the FDA, the LAL test became the first *in vitro* replacement for an animal toxicity test. Its widespread use today, accompanied by an excellent safety record for drug products with respect to pyrogenicity is testimony to the fact that endotoxin is indeed the only pyrogen of concern in products produced under cGMP. Surprisingly however, the non-endotoxin pyrogen controversy of the 1980's has never completely disappeared. Beginning in 1989 *in vitro* "pyrogen" tests began to appear in the literature.<sup>12,13,14,15,16</sup>

These assays were based on the fact that mammalian monocytes, when stimulated by endotoxins and/or other pyrogens secrete endogenous pyrogen (interleukin-1B) and other cytokines (e.g. IL-6, TNF a, and PGE2). Thus, by using macrophages or blood containing macrophages from the drug target (e.g. man), a species specific pyrogen test was possible. Given that the PT still remains a required test at some point in the life cycle of a new drug, a species-specific test, especially one that eliminates the need to sacrifice animals and is sensitive to non-endotoxin pyrogens, is welcome.

It is important to note, however, that the monocyte assays have not yet been adequately characterized or validated and the suggestion that they become pharmacopeal tests may be premature. As in the examples of contaminated LTA discussed earlier, some of the data supporting use of the monocyte assay is suspect.<sup>17</sup>

The most glaring deficiency of these assays is the absence of a valid non-endotoxin "pyrogen" standard. All of the current *in vitro* assays use endotoxin as a standard. Until a consensus non-endotoxin pyrogen standard(s) is available, the utility of these assays (including the PT for which no standard has ever been required) remains unknown. Another drawback of the monocyte assay is the requirement for fresh, whole human blood or collected monocytes. As with all biological systems, a great degree of variability exists. For example, in a study of 131 normal blood donors, endotoxin content averaged  $0.151 \pm 0.113$  EU/ml.<sup>18</sup> Although 73% of this group had no detectable endotoxin, a substantial number did. Thus, until the variability with respect to endotoxin presence can be defined and controlled, not to mention the innate ability of certain donors to neutralize endotoxin, the monocyte assay using fresh human blood will remain a research tool at best.<sup>19,20</sup> There is some encouragement in that cell culture *in vitro* pyrogen assays have been described. As with the LAL test however, scrupulous detail will need to be maintained with respect to keeping these systems endotoxin-free (i.e. glass ware, tissue culture media, transfer devices, etc.). Finally, as was evidenced by the experiments of Dinarrello et al concerning LAL-RM, the extracts under test, while not eliciting a positive response, were toxic to the cell culture system he employed.<sup>21</sup> Thus, the concept of a positive product control needs to be introduced to eliminate false negatives. Unlike LAL where the control is endotoxin, a valid control for an *in vitro* pyrogen test would need to be representative of all anticipated non-endotoxin pyrogens as these would not be expected to interact with test components in a manner similar to endotoxin. Finally, it should be noted that in order to obtain sensitivities similar to the LAL test even when using endotoxin as the test "pyrogen", a typical *in vitro* pyrogen test needs a preliminary incubation of 12-24 hrs (cytokine expression) followed by the assay for the cytokines themselves which can take up to 4 hours. To keep cells alive for this period of time requires a CO<sub>2</sub> incubator and careful attention to avoid contamination. Future UPDATES will provide additional details from our own research investigations with these assays.

### LAL Reactivity to Fungal Glucans

One of the major criticisms of the LAL assay was that it was not entirely specific for endotoxin. We now know that (1→3)-β-D-glucans of fungal origin are reactive with LAL (depending on formulation) down to the picogram level (LAL UPDATE, Vol. 19, No. 3, November 2001). Although it is not yet clear whether all LAL-reactive glucans are pyrogenic, i.e. elicit the expression of cytokines, when injected into warm-blooded animals or are introduced into specific cell cultures, it is clear that most (1→3)-β-D-glucans are potentially biologically active and represent a clear case of an adulterated (i.e. contaminated) product when present in an otherwise cGMP-prepared pharmaceutical drug or device. Thus it is entirely fortuitous that an LAL formulation containing uninhibited factors C and G, will detect the most common bacterial and fungal component contaminants. On the practical side, it is now quite easy to differentiate (and quantify) a positive LAL test into its glucan and/or endotoxin components using new products from ACC.

### LAL vs. The *In Vitro* Pyrogen Assays for Endotoxin

Since most of the publications to date describing the *in vitro* Pyrogen assays use endotoxin as the "pyrogen of choice", it is quite easy to compare them to the BET. Although the LAL test easily outperforms any of the described *in vitro* Pyrogen tests with respect to sensitivity, specificity, time, ease of use, cost, and reproducibility; three attributes of an *in vitro* Pyrogen test have been touted as making it superior to the LAL test. These are: 1) species specificity, i.e. human, rabbit, rat, etc.; 2) horseshoe crab protection, i.e. fewer horseshoe crabs would be needed due to a reduction in LAL testing; and, 3) endotoxins on the surface of devices could be detected. I have no argument with the first attribute other than to say that the endotoxin limit concept is most likely very conservative. Thus, regardless of the relative "potency" of different species of endotoxin in different species of animals, there is sufficient

data in the literature to show that a drug/device that passes a valid LAL test will not be pyrogenic in humans or other animals. In fact, human pyrogenicity using the RSE has shown pyrogenic dose identical to that in rabbits.<sup>22</sup> I also have no argument with the second statement except to say that LAL production has had no detectable impact on horseshoe crab populations. Horseshoe crabs designated for biomedical (i.e. LAL) use have traditionally been returned to their native environment. ACC has also been working diligently to develop assays that use less LAL (e.g. the new PK system uses ½ the amount of reagent needed for other LAL assays) and ACC has produced an endotoxin assay that uses all synthetic components. In addition, ACC has been active in promoting the banning of the horseshoe crab for bait use and has developed a substitute bait/attractant from the by-products of LAL manufacture. The third statement is perhaps the most interesting. It was argued that the *in vitro* Pyrogen test is a much more accurate test since it can be incubated in contact with a medical device to measure endotoxin which may be adsorbed to the device. While it is true that the current accepted LAL procedure for measuring endotoxin contamination in devices employs an assay of a rinse which may not remove all (in extreme cases none may be removed) adsorbed endotoxin, *in situ* LAL assays, i.e. assays where devices are incubated in direct contact with the LAL reagent have been described in the literature and work quite nicely.<sup>23,24</sup> In fact, ACC has recently completed a study of implants which exhibited negative endotoxin when rinsed in the traditional manner but which had significant LAL reactivity when the device was incubated *in situ*, i.e. bathed in the LAL reagent. We plan to publish this procedure in the near future. It is anticipated that the FDA will recognize the advantages of such an *in situ* technique for certain implants and adapt their regulations accordingly.

## References

1. Federal Register 42:57749, November 4, 1977.
2. USP XXIV. United States Pharmacopeial Convention, Inc. Rockville, MD. (2000).
3. Müller-Calgan, H., "Experiences with comparative examinations for pyrogens by rabbit pyrogen test versus the LAL test." In Watson S. W., Levin, J. & Novitsky T.J. (Eds.), Endotoxins and Their Detection with the Limulus Amebocyte Lysate Test. Alan R. Liss, Inc., New York, NY, pp.343-356 (1982).
4. Hochstein, H.D., "The LAL test versus the rabbit pyrogen test for endotoxin detection." Pharmaceutical Technol. 1:124-129 (1987).
5. Pearson, F.C. III, M.E. Weary, and R. Dabbah, "A corporate approach to in-process and end-product testing with the LAL assay for endotoxin." In Watson S. W., Levin, J. & Novitsky T.J. (Eds.), Endotoxins and Their Detection with the Limulus Amebocyte Lysate Test. Alan R. Liss, Inc., New York, NY, pp.231-246 (1982).
6. Twohy, C.W., A. P. Duran, and T. Munson, "Endotoxin contamination of parenteral drugs and radiopharmaceuticals as determined by the Limulus amebocyte lysate method." J. Parenteral Sci. Technol. 38:190-201 (1984).
7. Seibert, F.B., "Pyrogens from an historical viewpoint." Transfusion 3:245-249 (1963).
8. Seibert, F.B., "Fever-producing substance found in some distilled waters." Am. J. Physiol. 64:90-104 (1923).
9. Pearson, F.C. III, Pyrogens. Endotoxins, LAL Testing, and Depyrogenation. Advances in Parenteral Sciences/2, Marcel Dekker, Inc. New York (1985).
10. Gao, J.J., Q. Xue, E.G Zuvanich, K. R. Haghi, and D.C. Morrison. Commercial preparations of lipoteichoic acid contain endotoxin that contributes to activation of mouse macrophages *in vitro*. Infection and Immunity 69:751-757 (2001).
11. Morath, S., A. Geyer, I. Spreitzer, C. Hermann, and T. Hartung. Structural decomposition and heterogeneity of commercial lipoteichoic acid preparations. Infection and Immunity. 70:938-944, (2002).
12. Hartung, T., S. Fennrich, A. Wendel, "Detection of endotoxins and other pyrogens by human whole blood." J. Endotoxin Res. 6:184 (2000).
13. Hartung, T., A. Wendel, "Detection of pyrogens using human whole blood." *In Vitro Toxicol.* 9:353-359 (1996).
14. Wendel, A., and T. Hartung, "Test for determining pyrogenic effect of a material." United States Patent 5,891,728 (1999).
15. Poole, S., S. Selkirk, B. Rafferty, A. Meager, R. Thorpe, and A. Gearing, "Assay of pyrogenic contamination in pharmaceuticals by cytokine release." Pharmeuropa 1:17-18 (1989).
16. Flint, O. "Editorial. Prize for new *in vitro* test." *In Vitro Toxicol.* 9:341(1996).
17. Wang, E., P.F. Jørgensen, M. Almlöf, C. Thiemeermann, S.J. Foster, A.O. Aasen, and R. Solberg, "Peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model." Infection and Immunity 68:3965-3970 (2000).

# CALENDAR OF EVENTS



18. Ketchum, P.A., J. Parsonnet, L.S. Stotts, T.J. Novitsky, B. Schlain, D.W. Bates, Investigators of the AMCC SEPSIS Project. Utilization of a chromogenic Limulus amoebocyte lysate blood assay in a multi-center study of sepsis. *J. Endotoxin Res.* 4:9-16 (1997).
19. Warren, H.S., T.J. Novitsky, P.A. Ketchum, P.F. Roslansky, S. Kania, and G.R. Siber. Neutralization of bacterial lipopolysaccharides by human plasma. *J. Clin. Microbiol.* 22:590-595 (1985).
20. Warren, H.S., T.J. Novitsky, P. Martin, P.F. Roslansky, and G.R. Siber. Endotoxin neutralizing capacity of sera from different patient populations assessed by the Limulus lysate test. In *Detection of Bacterial Endotoxins with the Limulus Amoebocyte Lysate Test*. Alan R. Liss, Inc., New York, NY, pp.341-348 (1987).
21. Pearson, F.C., J. Bohon, W. Lee, G. Bruszer, M. Sagona, R. Dawe, G. Jakubowski, D. Morrison, and C. Dinarello. Comparison of chemical analyses of hollow-fiber dialyzer extracts. *Artificial Organs* 8:291-298 (1984).
22. Hochstein, H.D., E.A. Fitzgerald, F.G. McMahon, and R. Vargas. Properties of US Standard Endotoxin (EC-5) in human male volunteers. *J. Endotoxin Res.* 1:52-56 (1994).
23. Ragab, A.A., R. VanDeMotte, S.A. Lavish, V.M. Goldberg, J.T. Ninomiya, C.R. Carlin, and E.M. Greenfield. Measurement and removal of adherent endotoxin from titanium particles and implant surfaces. *J. Orthopaedic Res.* 17:803-809 (1999).
24. Harris, N.S., and R. Feinstein. The LAL-bead assay for endotoxin. In *Biomedical Application of the Horseshoe Crab (Limulidae)*, Alan R. Liss, New York, pp. 265-274 (1979).

## JULY 2002

### July 16-18

#### LAL Training Courses

*Introductory, Advanced, and Laboratory Training*  
ACC Falmouth Facility  
Falmouth, MA

### July 22-24

#### Biomedical Focus

*Radisson South*  
Bloomington, MN

## SEPTEMBER 2002

### September 12-13

#### LAL Training Courses

*Hemodialysis Training*  
ACC Falmouth Facility  
Falmouth, MA

#### Customer Service:

(800) LAL-TEST  
(508) 540-3444

#### Technical Service:

(800) 848-3248  
(508) 540-3444

#### Website Address:

[www.acciusa.com](http://www.acciusa.com)

©2002 Associates of Cape Cod, Inc.

All rights reserved.

Printed on recycled paper. 



*Creating New Horizons in Endotoxin Testing*

704 Main Street <sup>na</sup> Falmouth, MA 02540

# LAL Update<sup>®</sup>

VOLUME 20, NO. 2