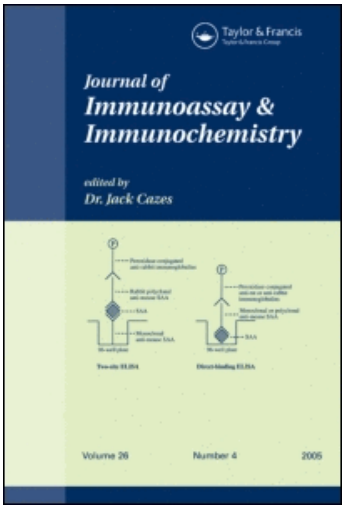

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The Detection of Pyrogens in Blood Products Using an *Ex Vivo* Whole Blood Culture Assay

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THE DETECTION OF PYROGENS IN BLOOD PRODUCTS USING AN EX VIVO WHOLE BLOOD CULTURE ASSAY

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ABSTRACT

Induction of interleukin-6 (IL-6) secretion by whole blood cultures (WBC) was used as an *in vitro* assay system for pyrogen-induced inflammatory reactions. The assay system was very sensitive to *Eschericia coli (E.coli)* endotoxin (< 10 pg/ml). The potential pyrogenic effects of human serum albumin (HSA), Fibronectin (Fn) and stabilised human serum (SHS) solutions were analyzed using this system. None of the products assayed had an effect on the sensitivity of the WBC assay. Spike recovery studies with isolated endotoxin, gram positive and gram negative bacteria showed that none of the products had an effect on the spike recovery of these pyrogenic substances. Good correlations were found between the WBC assay and the rabbit assay for pyrogens for all the production batches tested. When these samples were analysed by the limulus amoebocyte lysate (LAL) assay, the LAL test gave anomalous results for 1 out of the 22 production batches tested. This batch gave a false negative result on the LAL assay and might be indicative of the inability of the LAL assay to detect pyrogens other than endotoxin.

(KEY WORDS: IL-6, pyrogen, endotoxin, fibronectin, albumin, serum)

INTRODUCTION

Pharmaceutical products intended for parenteral use must be free of pyrogens, i.e. endotoxin, Gram-positive bacteria, fungi and viruses. Humans are particularly sensitive

to bacterial products and nanogram quantities of bacterial lipopolysaccharides induce leucocytosis, hypoferremia, and fever in humans (1,2,3). Pyrogenicity is usually tested in the rabbit pyrogen test, the Limulus Amoebocyte Lysate (LAL) test, or by *in vitro* cell culture assays. The rabbit pyrogen test is based on the fact that there is a temperature increase in animals following intravenous injection of pyrogen (2). Rabbits have a sensitivity for pyrogens comparable to humans and the rabbit pyrogen test recognise all kinds of pyrogens (4). The rabbit test is thus ideal for the detection of pyrogens in pharmaceutical products. However it is expensive and impractical for assaying large numbers of samples. The LAL test is based upon the observation that pyrogen addition to the LAL reagent results in the gelation of the reaction mixture. The LAL test however does not detect pyrogens other than endotoxin (5,6). The endotoxin activated LAL reaction has been shown to be a valuable tool for testing raw materials, components in various stages of manufacture and for quality control of finished products (7-13).

The *in vitro* cell culture assay is based on the fact that pyrogens stimulate peripheral blood monocytes, macrophages and Kupffer cells to produce cytokines (14). Several studies have shown that the amount of cytokine released by the cultured cells is dependent on the endotoxin concentration of the culture medium. Several cell culture assay systems have been used to detect pyrogens in pharmaceutical preparations. They all employ cultured cells incubated in the presence or absence of endotoxin. At the end of the incubation period the cytokine/s secreted into the culture medium are assayed.

The initial work on cell culture assays was done with isolated peripheral blood monocytes (PBM). At the end of the culture period, supernatants were collected and injected into animals. Fever produced by the culture supernatant was indicative of pyrogens in the sample (15,16). Subsequent developments include the use of *in vitro*

biological assays for specific interleukins (17,18), the use of specific immuno-assays for cytokines (17-20) and the use of continuous monocytic cell lines instead of primary PBM cultures (19,20), and the use of unfractionated blood cells (21).

None of the cell culture assays have been registered with medical authorities for control purposes of pharmaceutical products. Guidelines for validating alternative tests for bacterial endotoxins have been published in the 1996 supplementary chapter of the British Pharmacopoeia (22). In brief: 'With the permission of the national authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monograph would be achieved if the official method were used. The presence of interfering substances should be tested for on samples of at least three production batches of product and the sensitivity of the assay in the presence and absence of product should not differ significantly. The sensitivity of the assay in the presence of product should not be less than 0.5 times and not more than 2 times the sensitivity of the assay in the absence of product. Samples of production batches that tested positive on the rabbit test for pyrogens should also test positive with the alternative test method for bacterial endotoxins'.

The aim of this study is to validate the WBC pyrogen assay as an alternative pyrogen assay to the rabbit pyrogen assay for blood products.

METHODS

ELISA for IL-6

Assays were carried out in 96 well plates (Nunc-Immuno plate, MaxiSorp). Wells were coated with 100 µl/well of rabbit anti-IL-6 (Sigma) diluted 1/1000 in saline by

overnight incubation at ambient temperature. The coating antibody was aspirated and the wells were blocked with 200 µl/well of 1 % rabbit serum (Highveld Biologicals, South Africa) diluted in saline for 30 minutes at ambient temperature. The plate was then washed once with saline containing 0,1 % Triton X100 (wash solution). Samples or standards were added at 100 µl/well and the plate was then incubated for 90 minutes at 37 °C. After washing the plate three times, 100 µl biotinylated rabbit anti-IL-6 (Sigma antibody biotinylated using the Boehringer Mannheim protein biotinylation kit) diluted to 1/200 in 1 % rabbit serum was added to each well and the plate was again incubated at 37 °C for 90 minutes. The plate was then washed three times followed by the addition of 100 µl avidin-peroxidase complex (Amdex, Denmark) diluted to 1/1000 with 1 % rabbit serum to each well. The plate was incubated for 20 minutes at room temperature and then it was washed four times. TMB substrate (Boehringer-Mannheim, Germany) was added at 100 µl/well and the plate was incubated at ambient temperature in the dark for 20 minutes. The chromogenic reaction was stopped by the addition of 100 µl/well of 0.2 M H_2SO_4. The optical densities were read on a plate reader at 450 nm.

Collection and culture of whole blood

Blood from healthy laboratory personnel, not on any medication, was collected by venipuncture into heparinised vacuum tubes. All donors were males between the ages 25 to 40 years with normal white blood cell counts. The blood was diluted with four volumes of RPMI medium. IF-γ (Boehringer-Mannheim, Germany) was added to a final concentration of 500 U/ml. Additives plus RPMI medium (final volume of 40 µl) was added to 500 µl of diluted blood into sterile culture tubes (Nunc) or 48 well culture plates (Nunc). The assay mixture was incubated at 37 °C for 18 hours. At the end of the

incubation period the cell supernatant was collected and assayed for IL-6 using the immuno-assay described above.

Preparation of samples for WBC assay

Liquid HSA at 200 g/l (WPBTS, South Africa) and liquid SHS at (WPBTS, South Africa) were assayed without any further treatment. Freeze dried Fn at 1 mg/vial (WPBTS, South Africa) was reconstituted in 1 ml water for injection. The Fn solution was diluted to 0.5 mg/ml with RPMI for WBC assays.

Pyrogen test using the WBC assay

Standards were prepared by the addition of 20 µl/tube of *E.coli* endotoxin standard (Kabi) plus 20 µl/tube RPMI. Sample tubes received 20 µl/tube of sample plus 20 µl/tube of RPMI. Spiked sample tubes received 20 µl/tube sample and 20 µl/tube of standard endotoxin. Each tube then received 500 µl diluted heparinised blood. The tubes were then capped and incubated at 37 °C for 18 hours. The culture supernatant was assayed for IL-6.

Comparison between the WBC, LAL and Rabbit assay for batches of pharmaceutical products

Comparative studies were done on batches of plasma fractionation products. The LAL assay was done using a endotoxin gelation assay kit (Pyrogent Assay Kit, Bio-Whittaker, USA), while rabbit pyrogen assays were done using the British Pharmacopoeia method.

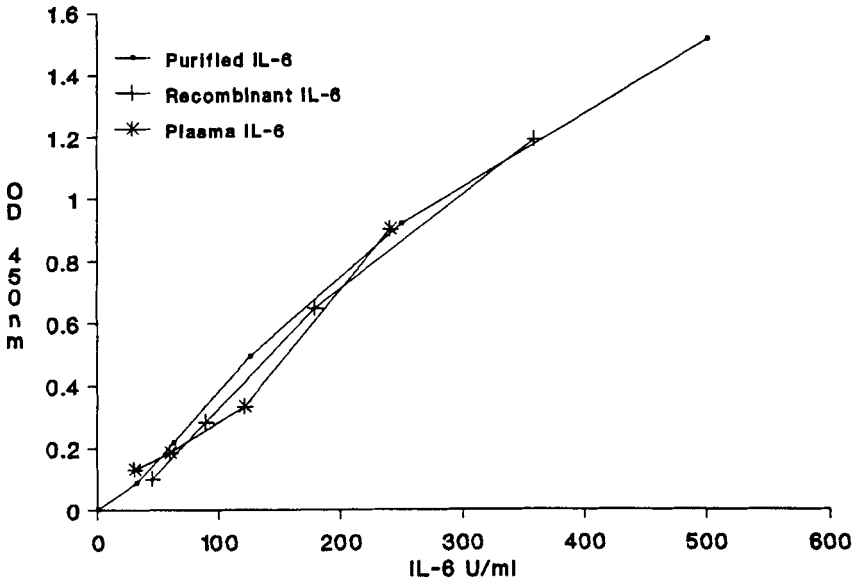

Figure 1: ELISA for IL-6
ELISA standard curves using rIL-6, plasma IL-6 and highly purified plasma IL-6.

RESULTS

The detection of IL-6 by ELISA

An in-house ELISA was developed to determine the concentration of IL-6 in culture supernatants and plasma samples. Studies done by us and also by other investigators have shown that problems are occasionally encountered when plasma IL-6 is assayed using a monoclonal antibody based ELISA (23). These problems were overcome by the use of polyclonal antibodies for both trapping and detection. The ELISA assay is linear with respect to IL-6 concentration between the range 3-100 U/ml. The standard curves for plasma IL-6 and highly purified plasma IL-6 were similar to that of the recombinant IL-6 standard used routinely in assays (Figure 1).

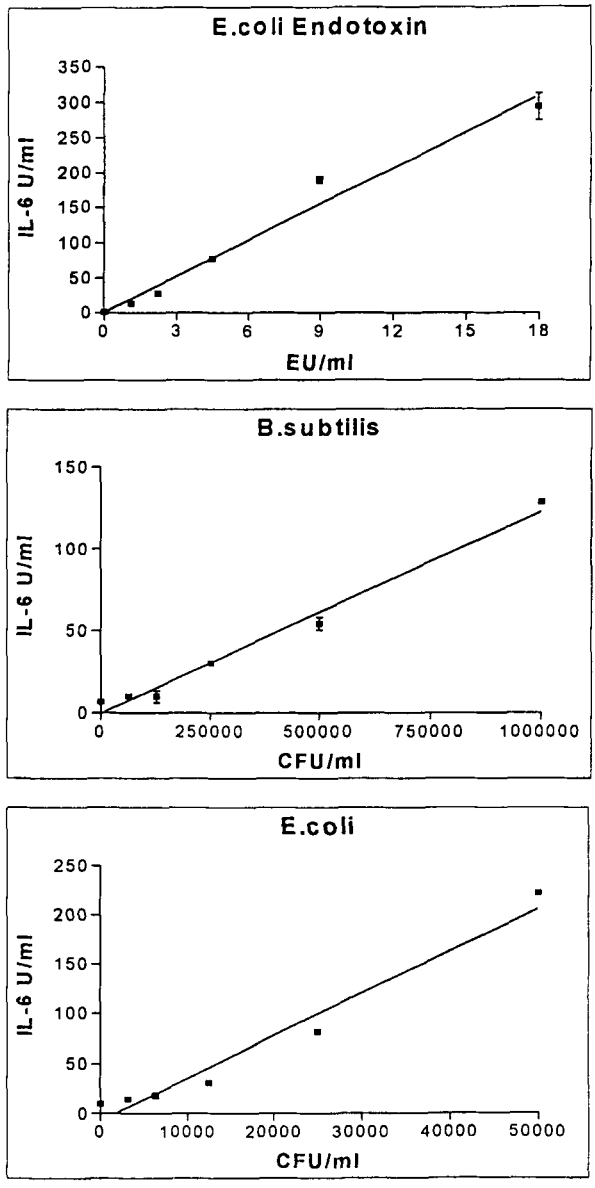

Figure 2: The effect of pyrogen concentration on IL-6 secretion by whole blood cultures. *Whole blood cultures were incubated in the presence of different pyrogens as indicated. The amount of IL-6 secreted after an 18 hour incubation at 37 °C was measured by ELISA.*

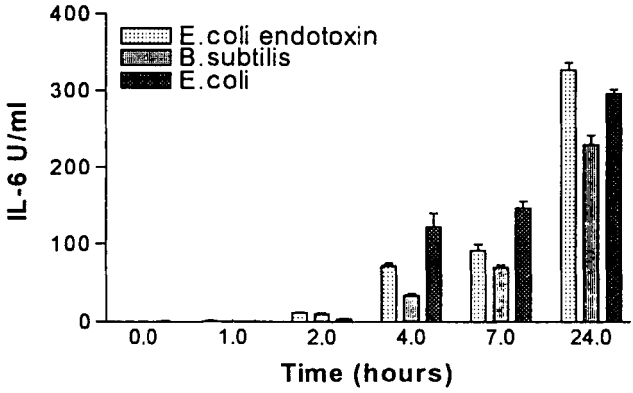

Figure 3: The effect of culture time on IL-6 secretion by whole blood cultures.
Whole blood cultures were innoculated with E.coli endotoxin (10 EU/ml), B.subtilis (500000 CFU/ml) or E.coli (25000 CFU/ml). IL-6 secreted by the cultures were measured at time periods as indicated.

The effect of pyrogen concentration and time of culture on IL-6 secretion

Blood cultures were inoculated with *E.coli* endotoxin, *E.coli* or *Bacillus Subtilis (B.subtilis)* at known concentrations. Results obtained shows that the amount of IL-6 secreted is dependent on the concentration of pyrogen added to the culture for all three pyrogens tested (Figure 2). The secretion was also time-dependent and IL-6 secretion induced by 20 EU/ml endotoxin can be detected as early as 4 hours after inoculating the culture with pyrogen. After 7 hours of incubation the level of IL-6 secreted by 1.25 EU/ml endotoxin was high enough to be assayed by the IL-6 ELISA. IL-6 secretion was monitored over 24 hours and at this point the IL-6 level in the culture supernatant was at its highest for all the pyrogens tested (Figure 3). Earlier studies done by us showed that the IL-6 levels secreted by WBC decreased after 12 hours in culture when neat blood was used. The dilution of blood with RPMI medium resulted in IL-6 being secreted over a longer period. The effect of variation between individuals was also investigated and it can

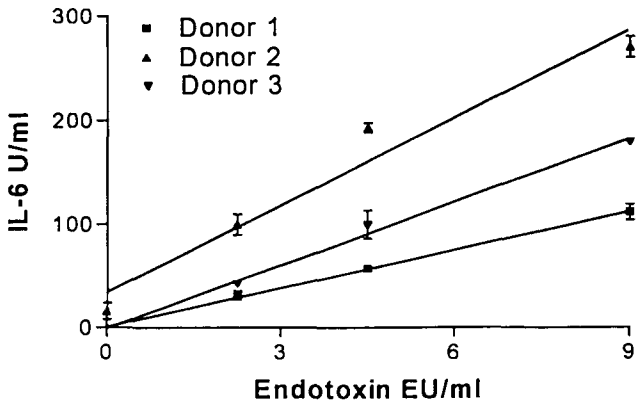

Figure 4: IL-6 secretion by whole blood cultures from different donors upon stimulation with *E.coli* endotoxin.

be seen that although there is great variation between the amount of IL-6 secreted by different individuals, there is always a linear relationship between IL-6 secretion and the amount of endotoxin added to the culture for endotoxin concentrations lower than 9 EU/ml (Figure 4). The slope of these curves are not dependent on the white blood cell count for individuals when the cell count is within the normal range and repeat experiments with cells collected from the same donor on different days gave similar results. These studies indicate that the slope of the curve is an inherent property of the cells from an individual to synthesise and release IL-6. All individuals tested thus far can detect *E.coli* endotoxin concentrations less than 1.5 EU/ml. Due to the variation of slopes for IL-6 secretion upon endotoxin stimulation, an endotoxin standard curve must be included for each experiment.

The effect of blood products on the sensitivity of the WBC assay for pyrogen

Samples of SHS, Fn and HSA production batches were tested for assay interference.

TABLE 1

The recovery of pyrogen spikes from SHS, Fn and HSA production batches using the WBC assay.

		% Spike recovery. Mean ± STD (n=4)		
Product	Batch	*E.coli* endotoxin	*B.subtilis*	*E.coli*
SHS	SS349	91 ± 13	95 ± 8	79 ± 10
	SS352p	96 ± 4	78 ± 7	97 ± 10
	SS352	90 ± 5	89 ± 5	99 ± 4
HSA	B282	94 ± 12	102 ± 4	61 ± 5
	B298	108 ± 6	89 ± 5	84 ± 10
	B284	113 ± 21	111 ± 15	95 ± 8
Fn	Fn31	115 ± 17	95 ± 33	N.D.
	Fn32	107 ± 2	72 ± 2	N.D.
	Fn35	116 ± 13	74 ± 1	N.D.

N.D. not done

Cultures were spiked with 10 EU/ml E.coli endotoxin, 500000 CFU/ml B.subtilis or 25000 CFU/ml E.coli.

The results obtained show that none of these products interferes with the assay system when endotoxin recovery in spiked samples was determined (Table 1). The endotoxin spike recoveries obtained varied between 90 % obtained for SHS batch SS352 and 116% for Fn batch Fn35. The standard deviations for endotoxin recovery of all the products tested were less than 12 %. The study was extended by including samples spiked with known concentrations of gram negative or gram positive bacteria. Gram negative and gram positive bacteria contain components other than endotoxin that are pyrogenic for mammals. The spike recovery for samples spiked with *B.subtilis* varied between 72 %

TABLE 2

The effect of endotoxin concentration on the rabbit pyrogen assay. Pyrogen negative batches were spiked with known concentrations of endotoxin and injected into rabbits as per protocol.

Product	Endotoxin	Temperature °C[a]	Pass/Fail
HSA	2.0 EU/ml	1.1	Pass
SHS	2.0 EU/ml	0.5	Pass
Fn	4.5 EU/ml	1.05	Pass

The injection protocols for the products were as follows:

Inject 3 ml of SHS at 50 mg/ml per kg rabbit.
Inject 3 ml of HSA at 200 mg/ml per kg rabbit.
Inject 1 ml of Fn at 0.5 mg/ml per kg rabbit.
[a] *Combined temperature change for three rabbits.*

and 111 %, and for those spiked with *E.coli* between 61 % and 97 % (Table 1). Variations in *B.subtilis* and *E.coli* spike recovery was greater than that found for endotoxin (Table 1). This is probably due to the more complex nature of whole bacteria as compared to purified endotoxin.

The determination of cut-off levels of endotoxin in HSA, SHS and FN

Cut-off levels of endotoxin in the different products were established using the rabbit assay for pyrogens. *E.coli* endotoxin was used for these studies as *E.coli* endotoxin gives WBC assay standard curves with $r \geq 0.98$ using linear regression analysis at endotoxin concentrations between 0 and 9 EU/ml. Initial experiments using the rabbit assay were done to establish the maximum concentration of the standard endotoxin preparation that will give a negative response in rabbits. The maximum level of endotoxin that results in a negative pyrogen response was 2 EU/ml (Table 2). Spiking of previously determined

TABLE 3

Comparison between the WBC assay, LAL assay and the rabbit pyrogen assay for production batches of biological products.

Product	Batch	WBC EU/ml	LAL	Rabbit Assay
Fn: 0.5 mg/ml	Fn3195	<0.5	Pass	Pass
	Fn3296	<0.5	Pass	Pass
	Fn3596	1.28	Pass	Pass
HSA: 200 mg/ml	B274	29.4	Fail	Fail
	B291	<0.5	Pass	Pass
	B293	<0.5	Pass	Pass
	B294	<0.5	Pass	Pass
	B295	<0.5	Pass	Pass
	B296	<0.5	Pass	Pass
	B297	<0.5	Pass	Pass
	B298	1	Pass	Pass
	B299	1.1	Pass	Pass
	B300S	1	Pass	Pass
	B301	<0.5	Pass	Pass
	B302	>20	Pass[a]	Fail
SHS: 50 mg/ml	SS349	0.7	Pass	Pass
	SS350	<0.5	Pass	Pass
	SS351	<0.5	Pass	Pass
	SS352	0.5	Pass	Pass
	SS353	<0.5	Pass	Pass
	SS354	0.6	Pass	Pass
	SS355	0.5	Pass	Pass

[a] *false negative*

pyrogen negative samples with known amounts of endotoxin showed that the SHS and HSA spiked with 2 EU/ml of our standard endotoxin preparation gave a negative response on the rabbit assay. The temperature increases for these samples (0.5 °C for SHS and 1.1 °C for HSA) are below the acceptable upper limit for pyrogen negative samples and these levels of endotoxin were subsequently used as maximum cut-off for these two products. Spiking of previously determined pyrogen negative samples with known amounts of endotoxin showed that Fn spiked with 4.5 EU/ml of standard endotoxin preparation gave a negative response on the rabbit assay. The temperature increase for these samples (1.05 °C for Fn) are below the acceptable upper limit for pyrogen negative samples and this level of endotoxin was subsequently used as maximum cut-off for Fn. The endotoxin cut-off levels for all three products are above the minimum detectable endotoxin concentration for the whole blood culture assay.

Comparison between the LAL, WBC and Rabbit assay for pyrogen

Results obtained for freshly prepared production batches of various biologicals were assayed for pyrogenicity using the three assay systems. *E.coli* endotoxin was used as standard for the WBC and LAL assays. WBC standard curves were calculated using linear regression analysis and r values for these standard curves were always ≥ 0.98. The WBC assay correctly predicted the pyrogenic status of 22 out of 22 freshly prepared batches of biologicals (Table 3). The LAL assay on the other hand showed one false negative when compared to the rabbit assay for pyrogens. The false LAL result was obtained for an albumin batch. This data is comparable to earlier reports where false results were obtained for albumin production batches using the LAL assay (19). These results might be due to the inability of the LAL assay to detect pyrogens other than endotoxins.

DISCUSSION

Previous studies done with isolated monocytes showed that sample concentration dependent interference was seen at high concentrations of HSA (19). The data obtained show that neither HSA, Fn nor SHS interfere with the WBC assay for pyrogen. This is probably due to the very low concentrations of product required to invoke reactions in the WBC system. The amount of sample used in previous studies accounted for 10 % of the final culture volume (19), while in the present study the sample volume is only 3.7 % of the final WBC culture volume. The minimum amount of endotoxin that can be detected by the WBC assay is 1.25 EU/ml. This is below the pyrogen cut-off level, as established by the rabbit assay, for the products under investigation (2 EU/ml is the maximum allowed level for HSA and SHS; 4,5 EU/ml is the maximum allowed level for Fn). Due to the linear relationship between endotoxin concentration and IL-6 secretion an unequivocal assessment can be made regarding the disposition of products once a cut-off maximum has been established for pyrogen levels in a product.

Good correlations were found between the WBC assay and the rabbit pyrogen assay for 22 out of the 22 freshly prepared production batches showed in this paper. Retrospective checks on old batches showed good correlations for 61 out of 63 production batches tested thus far. The two anomalous batches are being investigated at present and indications are that the pyrogenic status of these products has changed upon storage (data not shown).

These results show that the WBC assay can accurately predict the pyrogenic status of biological pharmaceutical products. The WBC assay, unlike the LAL assay, can detect pyrogens other than endotoxin from gram negative bacteria and has the advantage over the rabbit assay in that it is more sensitive and can be easily adapted to assay large numbers of samples.

In conclusion, the WBC assay for pyrogens complies with the requirements as set out in the British Pharmacopoeia for an alternative endotoxin assay and we strongly advocate its use as alternative pyrogen assay.

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