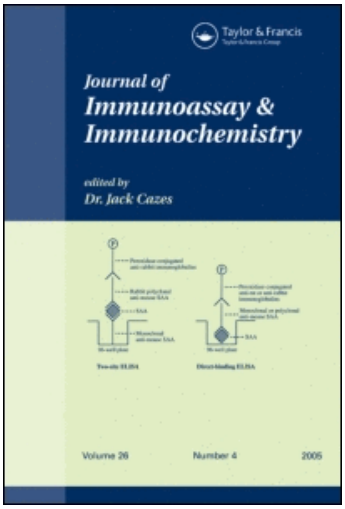

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Edmund J. Pool[a]; Gamieda Johaar[a]; Sandra James[a]; Ignatius Petersen[a]; Patrick Bouic[b]
[a] Western Province Blood Transfusion Service, Parow, South Africa [b] Department of Medical Microbiology, University of Stellenbosch, Tygerberg, South Africa

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DIFFERENTIATION BETWEEN ENDOTOXIN AND NON-ENDOTOXIN PYROGENS IN HUMAN ALBUMIN SOLUTIONS USING AN EX VIVO WHOLE BLOOD CULTURE ASSAY.

Edmund J. Pool[a], Gamieda Johaar[a], Sandra James[a],
Ignatius Petersen[a], Patrick Bouic[b]
[a] Western Province Blood Transfusion Service, Parow, South Africa
[b] Department of Medical Microbiology, University of Stellenbosch, Tygerberg, South Africa

ABSTRACT

Purified E.coli endotoxin, Gram negative bacteria and Gram positive bacteria induce IL-6 secretion by whole blood cultures (WBC's). Polymyxin B at concentrations greater than 2 U/ml completely inhibits IL-6 secretion caused by 10 EU/ml of endotoxin. Polymyxin B has no effect on IL-6 secretion by WBC's in the absence of endotoxin. The inhibition of endotoxin induced IL-6 secretion is Polymyxin B concentration dependent at concentrations less than 1 U/ml. IL-6 induction caused by E.coli is only partially inactivated by 8 U/ml Polymyxin B. Polymyxin B has no effect on IL-6 secretion caused by B.subtilis. Two pyrogenic batches of human serum albumin (HSA), as tested by the rabbit assay for pyrogens, were also investigated. Polymyxin B at 4 U/ml inhibits less than 40 % of IL-6 secretion caused by these pyrogenic HSA batches. All the endotoxin activity in HSA samples spiked with purified endotoxin is inhibited by Polymyxin B indicating that HSA does not protect endotoxin against Polymyxin B inhibition. These results indicate that the pyrogenicity of these HSA batches are caused by Polymyxin B inhibitable and non-inhibitable fractions.

This study shows that pyrogenic substances other than endotoxin can contaminate batches of pharmaceutical products and that results obtained using the Limulus Amoebocyte Lysate (LAL) assay does not necessarily indicate the pyrogenic status of pharmaceutical products.

The WBC assay for pyrogens, having a broader sensitivity range than the LAL assay, is a better indicator of the pyrogenic status of pharmaceutical products.

[KEY WORDS: pyrogen, IL-6, Polymyxin B, endotoxin, non-endotoxin, pharmaceuticals]

Corresponding author: Prof. P. Bouic, Department of Medical Microbiology, University of Stellenbosch, P. O. Box 19063, Tygerberg 7505, South Africa. Tel. 027 21 9384035; fax 027 21 9384005

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 leukocytosis, 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 endotoxin activated LAL reaction has been shown to be a valuable tool for testing raw materials, components at various stages of manufacture and for quality control of finished products (7, 8, 9, 10, 11, 12, 13). The LAL test however does not detect pyrogens other than LPS (5,6).

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). The ability of endotoxin to cause fever in rabbits and induce cytokine secretion *in vitro* are due to the strongly cationic lipid A moiety of the molecule (15). Polymyxin B and other cationic antibiotics have been shown to be very effective in inhibiting the biological activities of endotoxin such as the induction of fever in rabbits (16,17) and the induction of cytokine secretion (18). Endotoxin act as chemoreceptor for Polymyxin B and this binding inhibits

endotoxin binding to its bio-receptors (21) that is required for endotoxin bio-activity. The aim of this study is to determine the effect of Polymyxin B on IL-6 induction by batches of HSA that we have previously tested to be highly pyrogenic using the rabbit and whole blood culture assays. However, these HSA batches gave anomalous LAL results.

METHODS

Collection and culture of Whole Blood

Blood from donors were collected and cultured as described previously (19). In brief, blood was collected from healthy volunteers into heparinised tubes. The heparinised blood was diluted 1/5 with RPMI medium containing IFN-γ. The blood was then cultured for 18 hours in the presence of samples or standards. At the end of the culture period the culture supernatants were assayed for IL-6 using an in-house ELISA assay as described previously (19).

The effect of Polymyxin B on IL-6 secretion

Samples and standards were prepared as before. Polymyxin B was added to cultures at final concentrations as indicated in the results section.

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.

RESULTS

The stimulation of IL-6 secretion by bacteria

Previous studies done by us have shown that IFN-γ addition to the culture medium increase the level of IL-6 production in the presence of pyrogens and subsequent assays were all done in the presence of 500 U/ml IFN-γ in the culture medium (data not shown). Both gram positive and gram negative bacteria induce IL-6 secretion by WBC's (Table 1) and this secretion by WBC's is dependent on the concentration of pyrogen in the culture (Figure 1). Although all bacteria tested thus far induce IL-6 secretion by WBC's, the bacterial concentration giving a positive IL-6 response vary greatly between the different gram negative and gram positive bacteria tested (Table 1). The response caused any single bacterium preparation is highly reproducible and the differences obtained for IL-6 secretion between the different bacteria are most probably due to the pyrogen content of the bacteria preparations.

The effect of Polymyxin B concentration on endotoxin induced IL-6 secretion by WBC

Polymyxin B inhibits endotoxin-induced IL-6 secretion by WBC's. Polymyxin B at concentrations greater than 2 U/ml completely inhibits the IL-6 secretion induced by 10 EU/ml endotoxin (Figure 2). Inhibition of IL-6 secretion occurs at concentrations of Polymyxin B lower than 2 U/ml and this is dose dependent. HSA has no effect on Polymyxin B inhibition of IL-6 secretion and the curves for the inhibition of IL-6 secretion in the absence and presence of HSA are parallel to one another (Figure 2).

TABLE 1

The Detection Limit for Bacteria using the WBC assay.

GRAM POSITIVE BACTERIA		GRAM NEGATIVE BACTERIA	
BACTERIUM	CFU	BACTERIUM	CFU
Bacillus stearothermophilus	$3,2x10^2$	*Eschericia coli*	$3,3x10^2$
Bacillus subtilis	$2,2x10^4$	*Klebsiella pneumonia*	$2,9x10^2$
Micrococcus luteus	$2,3x10^4$	*Proteus vulgaris*	$1,7x10^2$
Staphylococcus aureus	$5,8x10^5$	*Pseudomonas aeruginosa*	$4,0x10^2$

Dilutions of bacteria were assayed for pyrogenicity. The level of bacteria giving the same response as 1,25 EU/ml endotoxin was regarded as the lowest detection limit for a specific bacterium

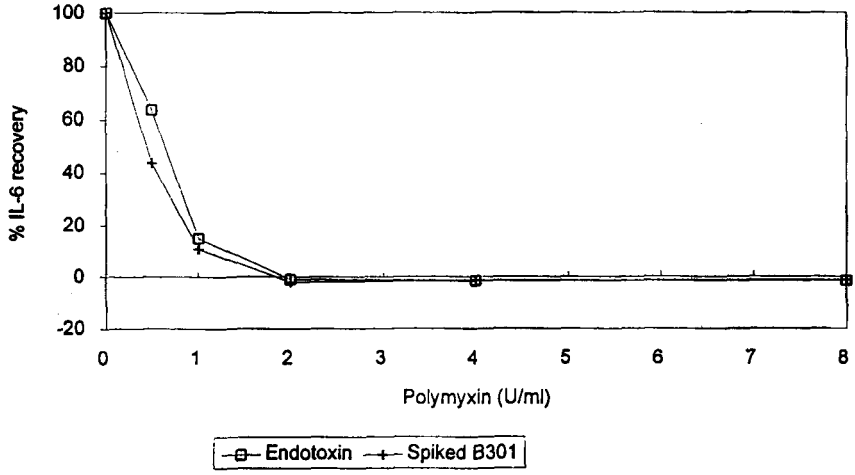

FIGURE 1: The effect of whole bacteria concentration on IL-6 secretion by whole blood cultures.
Whole blood cultures were incubated in the presence of different bacteria concentrations as indicated. The amount of IL-6 secreted after an 18 hour incubation at 37 °C was measured by ELISA.

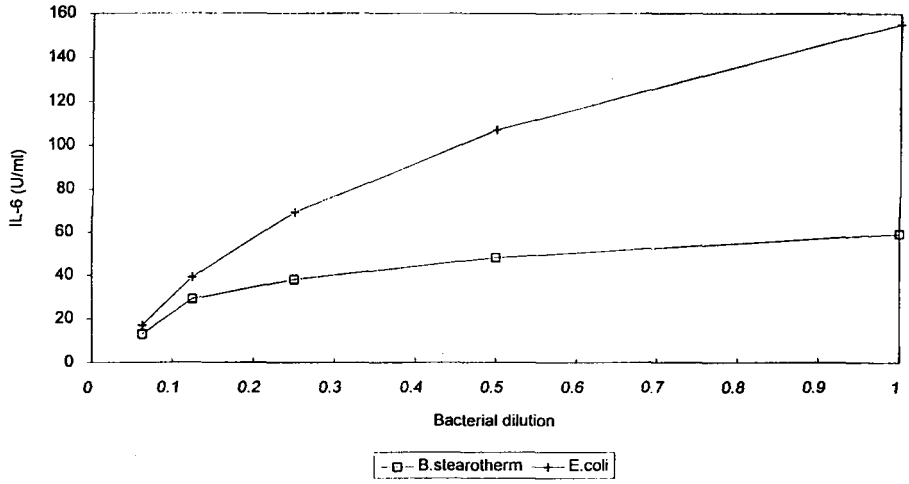

FIGURE 2: The effect of Polymyxin B concentration on endotoxin induced IL-6 secretion.
Whole blood cultures were incubated in the presence of 10 EU/ml endotoxin or HSA Batch B301 spiked with 10 EU/ml endotoxin. Polymyxin B was added to the cultures at concentrations as indicated. The amount of IL-6 secreted after an 18 hour incubation at 37 °C was measured by ELISA.

The effect of Polymyxin B on whole bacteria induced IL-6 secretion

Unlike the observation using endotoxin, Polymyxin B does not inhibit all the IL-6 inducing ability of whole bacteria (Table 2). Only 33 % of IL-6 activity induced by heat inactivated Gram negative bacteria can be inhibited by Polymyxin B at a concentration of 8 U/ml. As shown in Table 2, Polymyxin B has no effect on IL-6 secretion induced by Gram positive bacteria (decrease in IL-6 < 10 % at all concentrations of Polymyxin B tested). This is consistent with earlier studies which showed that Polymyxin B inhibits only the endotoxin fraction of gram negative bacteria and that gram negative bacteria contain other pyrogenic entity/ies that are far more potent than endotoxin in stimulating IL-6 secretion by cultured cells (20).

TABLE 2

The Effect of Polymyxin B on Whole Bacteria Induced IL-6 Secretion.

GRAM POSITIVE BACTERIA *B.subtilis*		GRAM NEGATIVE BACTERIA *E.coli*	
Polymyxin B (U/ml)	% IL-6 recovery	Polymyxin B (U/ml)	% IL-6 recovery
0	100	0	100
0.5	96	0.5	99
2	98	2	89
8	93	8	77

Whole blood cultures were incubated in the presence of bacteria. Different concentrations of Polymyxin B were added to the cultures. At the end of the incubation period the culture supernatants were assayed for secreted IL-6.

TABLE 3

Comparative Pyrogen Assays for B302 and B274.

Assay	LAL	Rabbit	WBC
Maximum	4 EU/ml	1,15 °C	2 EU/ml
B274	8 EU/ml	4,34 °C	> > 20 EU/ml
B302	Variable. Passes and failures	5.46 °C	> > 20 EU/ml

The effect of Polymyxin B on pyrogenic HSA induced IL-6 secretion

Two production batches of HSA, namely B274 and B302, that were tested pyrogenic by both the rabbit and the WBC assay (Table 3) were investigated for Polymyxin B inhibitable IL-6 inducing activity. Results (figure 3) show that Polymyxin B inhibits only 24 % IL-6 inducing activity of B302 and only 35 % IL-6 inducing activity of B274. This indicates that these batches of HSA contain pyrogenic substance/s other than endotoxin.

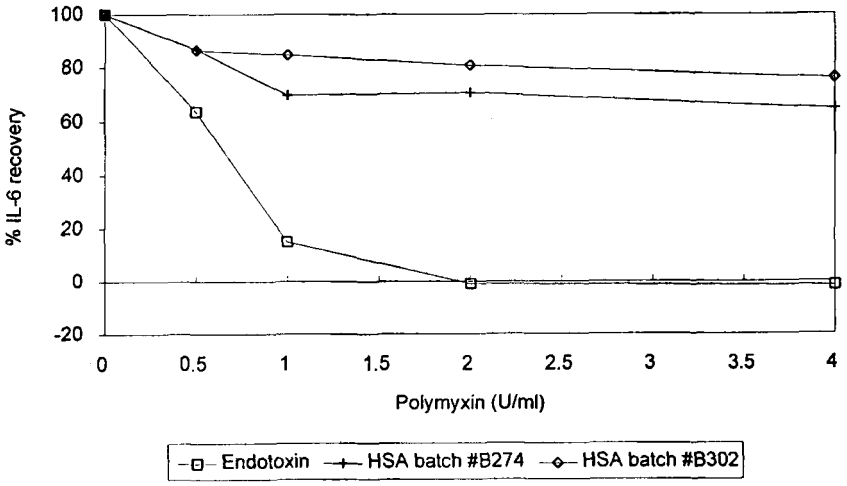

FIGURE 3: The effect of Polymyxin B concentration on pyrogenic HSA induced IL-6 secretion.
Whole blood cultures were incubated in the presence of pyrogenic HSA batches B274 and B302 or control HSA batch 301 spiked with 10 EU/ml endotoxin. Polymyxin B was added to the cultures at concentrations as indicated. The amount of IL-6 secreted after an 18 hour incubation at 37 °C was measured by ELISA.

Studies using the LAL assay for endotoxin confirms these results. All the IL-6 inducing activity in the control batch (B301 spiked with 20 EU/ml endotoxin) was inhibited at 2 U/ml Polymyxin B.

DISCUSSION

The WBC assay detects endotoxin and both gram negative and gram positive bacteria. The present study shows that all the IL-6 inducing activity of endotoxin can be inhibited by the addition of Polymyxin B to the culture medium. None of the IL-6 inducing activity produced by gram positive bacteria and only a fraction of the IL-6 inducing activity of

gram negative bacteria are inhibited by Polymyxin B. This observation makes Polymyxin B a useful tool to differentiate between endotoxin and non-endotoxin pyrogenic contaminants in pharmaceutical preparations. This is a very important factor for the assessment of the feasibility of "cleaning up" of pyrogenic batches. Batches of pharmaceutical products contaminated with endotoxin as the only pyrogen can be depyrogenated easily by Polymyxin B affinity chromatography or by DEAE ion exchange chromatography, whereas non-endotoxin contaminants require more sophisticated methods for depyrogenation.

Previous studies done by us have shown that some pyrogenic batches of HSA cannot be detected by the LAL assay, or that the endotoxin concentrations obtained for these batches do not agree with data obtained for the rabbit pyrogen assay (19). Two such batches have been assayed using Polymyxin B and data obtained showed that only a small percentage of the pyrogenic activity of these batches (24 % and 35 % respectively) was due to endotoxin. Studies have shown that only a small fraction of the IL-6 inducing activity of B274 can in fact be removed by Polymyxin B chromatography (data not shown). The remainder could not be removed by further Polymyxin B affinity chromatography steps. The other pyrogenic entities are Polymyxin B insensitive and will probably not be detected by the LAL assay. Assaying of samples from these batches by LAL during depyrogenation will not detect the non-endotoxin pyrogens that might remain in the batch. On the other hand the rabbit pyrogen assay is not feasible for assaying the large numbers of in-process samples required during depyrogenation. The WBC assay detects both endotoxin and non-endotoxin contaminants in pharmaceuticals. It can also be easily adapted for assaying large numbers of samples and can be used as a cheaper and more user friendly alternative to the rabbit assay for monitoring and assessing depyrogenation of pharmaceuticals.

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