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Comparative evaluation of the human whole blood and human peripheral blood monocyte tests for pyrogens

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Abstract

Two different in vitro tests for pyrogens, using human peripheral blood monocytes (PBMNC) and diluted whole blood (WBC), respectively, were applied to different classes of parenteral medicinal products. Many of these products did not have a specified endotoxin limit concentration that was established as the maximum valid dilution to comply with the test. The results of the in vitro tests for pyrogens were compared with the results from the *Limulus* amoebocyte lysate (LAL) and rabbit pyrogen tests. The Second International Standard for endotoxin was used to calibrate all of the assays and the International Standard for IL-6 was used to calibrate the IL-6 ELISA which provided the readout for the in vitro tests for pyrogens. Preparatory tests were conducted to ensure that the "criteria for validity and precision of the standard curve" were satisfied and that the drugs being tested did not interfere in the tests. The PBMNC/IL-6 test had a detection limit of 0.06 EU/ml and spike recoveries were 62–165%. The whole blood/IL-6 test also had a detection limit of 0.06 EU/ml and spike recoveries were 58–132%. The application to the detection of non-endotoxin pyrogens needs to be evaluated in more detail, but the two in vitro tests for pyrogens showed good agreement overall, both with each other and with the LAL test and the rabbit pyrogen test for the detection of endotoxins.

Keywords: Blood; IL-6; In vitro test; Pyrogens; Monocytes; Quality control

1. Introduction

Parenteral pharmaceutical products must be free of pyrogens from Gram-negative or -positive bacteria, fungi and viruses. The pyrogens most frequently encountered in pharmaceutical products are bacterial endotoxins (lipopolysaccharides, LPS) from the cell walls of Gram-negative bacteria (Mascoli and Weary, 1979a, 1979b). Humans are particularly sensitive to bacterial products and these LPS induce the whole cascade of defence mechanisms which comprise inflammation and fever (Fennrich et al., 1999). There are two pharmacopoeial tests for pyrogenic contamination: the rabbit pyrogen test and the *Limulus* amoebocyte lysate (LAL) test. The rabbit pyrogen test involves measuring the rise in body temperature evoked in rabbits by the intravenous injection of the solution being examined. The rabbit test uses experimental animals, is costly and is not quantitative. The principle of the LAL test is the extracellular coagulation of the blood of *Limulus polyphemus* (Levin and Bang, 1964); this test is more sensitive but detects only LPS from Gram-negative bacteria and can give false negative and false positive results (Poole et al., 1988; Ray et al., 1990; Taktak et al., 1991; Fennrich et al., 1999).

In view of the shortcomings of the rabbit pyrogen test and the LAL test, in vitro pyrogen tests that utilise the exquisite sensitivity to exogenous pyrogen

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of human monocytes have been proposed (Duff and Atkins, 1982; Dinarello et al., 1984; Poole et al., 1988; Poole et al., 1989; Hansen and Christensen, 1990). Human monocytes react to the presence of endotoxins by increasing the release of interleukin (IL)-6 and IL-1β. Initially, cytokines released from separated leukocytes (Dinarello et al., 1984) or leukocytic cell lines (Poole et al., 1988) were used to detect pyrogens. Cell culture tests produced comparable results to the LAL test and the rabbit pyrogen test, with fewer false positives than the LAL test (Eperon et al., 1997). However, the isolation of monocytes/leukocytes from whole blood is labour-intensive and time-consuming and does not guarantee the isolation of cells in a non-activated state.

To obviate the disadvantages of test systems based upon isolated white blood cells or monocytic cell lines, a simple test system has been proposed that utilises the stimulation by pyrogen/LPS of human whole blood to produce either IL-1β (Hartung and Wendel, 1996; Fennrich et al., 1999) or IL-6 (Pool et al., 1998; Poole and Gaines Das, 2001). The whole-blood in vitro pyrogen test offers the considerable advantage of not requiring the isolation of the cells and, moreover, is carried out in the presence of all the blood constituents with which monocytes usually reside. The ethical and safety aspects related to the use of human blood are considered and accepted in the context of the replacement and refinement of the pyrogens assays of the parenteral preparations.

The aims of the present study were, firstly, to evaluate the novel in vitro pyrogen assays based on the culture of human peripheral blood monocytes and human whole blood cells, and, secondly, to compare these with the LAL test and the in vivo rabbit pyrogen test for the screening of parenteral pharmaceutical products, with a view to their evaluation as possible alternatives to the current tests for pyrogens/endotoxin.

2. Materials and methods

2.1. Materials

2.1.1. Reagents, standards and antibodies

Second International Standard bacterial endotoxin (WHO 94/580, 10 000 EU/vial), International Standard human recombinant interleukin-6 (WHO 89/548, 1 μg/100 000 IU/ampoule), goat polyclonal anti-IL-6 antibodies and biotinylated goat polyclonal anti-IL-6 antibodies were kindly donated by the National Institute for Biological Standards and Control (NIBSC) (Herts, UK). Avidin-horseradish-HRP was from Dako (Glostrup, Denmark). Tween-20, *O*-phenylenediamine (OPD) and Histopaque-1077 were purchased from Sigma (St. Louis, MO, USA). RPMI-1640 growth medium and heat-inactivated foetal calf serum (HIFCS) were from Gibco (Grand Island, NY, USA). *Limulus* amoebocyte lysate reagent, 0.06 EU/ml was from Cape Code (Falmouth, MA, USA). Heparin (5000 IU/ml) was from Roche (São Paulo, SP, Brazil). *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 10231) and *Staphylococcus aureus* (ATCC 6538P) from the Industrial Pharmacy department (Santa Maria, RS, Brazil). Parenteral pharmaceutical products were used, in some cases different batches of the same product, all within their period of validity. Other reagents and plasticware were purchased as sterile and pyrogen-free and glassware was baked at 250 °C for 1 h prior to use.

2.2. Methods

2.2.1. Rabbit in vivo pyrogens test

The rabbit pyrogens test was carried out using healthy New Zealand White rabbits, injecting the maximum volume of 10 ml per kg in accordance with the specifications of the USP (USP 25, 2001).

2.2.2. LAL test for endotoxins

The LAL gel cloth test was carried out in accordance with the requirements of the Pharmacopoeias (USP 25, 2001; Ph. Eur., 2002). Preparatory tests were conducted to ensure that the "criteria for validity and precision" were satisfied and that the drugs being tested did not themselves interfere in the tests. Interference testing on the products was carried out according to the harmonised Pharmacopoeial LAL method.

2.2.3. Enzyme-linked immunosorbent assay (ELISA) of IL-6

The ELISA of IL-6 was carried out according to a previously published method (Taktak et al., 1991) and the detection limit calculated as 3.3× the standard deviation of the response divided by the slope of the calibration curve (Poole and Gaines Das, 2001).

2.2.4. Human peripheral blood monocytes cell culture assay (PBMNC)

Human blood monocytes were isolated from buffy coat residues of heparinised peripheral blood from healthy donors by density gradient centrifugation using Histopaque-1077 and used immediately after the isolation. The heparinised peripheral blood was diluted 1:1 with RPMI-1640 and 30 ml of this solution was layered onto 15 ml of Histopaque-1077 in a 50 ml test-tube and centrifuged at $400 \times g$ for 40 min. The upper layer of plasma was discarded and the PBMNC layer at the interface of the plasma and histopaque was removed, washed twice in RPMI-1640 (250 g, 10 min) and resuspended at 2×10^6 cells/ml in culture medium (RPMI-1640) containing 2% HIFCS. The PBMNC assay was carried out in 96-well microtitre plates as independent assays with four replicates for each of the three donors. Samples and endotoxin standard were added at 50 μl per well, respectively. RPMI-1640 containing 2% HIFCS was added to each well at 100 μl per well and 100 μl per well of isolated monocytes cell suspension, at 2×10^6 cells/ml, were added to achieve a final volume of 250 μl. The plate was incubated at 37 °C for 16–24 h in an atmosphere of 5% CO_2 in humidified air. Finally, the cell supernatant was collected and assayed for IL-6 by ELISA and the results combined.

2.2.5. Human whole blood cell culture assay (WBC)

Immediately after venous puncture, heparinised blood samples from three healthy donors were diluted 1:3 with 0.9% (w/v) saline solution. The assay was carried out in 96-well plates with four replicates. Samples and endotoxin standard were added at 50 μl per well, respectively. Saline solution was added to each well at 50 μl per well, and 150 μl per well of the diluted whole blood were added to give a final volume of 250 μl. The plate was incubated at 37 °C for 16–24 h in an atmosphere of 5% CO_2 in humidified air. Finally, the cell supernatant was collected and assayed for IL-6 by ELISA.

2.2.6. Test for interfering factors

This test was performed on the PBMNC and WBC by incubating the PBMNC or blood from three different donors with endotoxin standard at concentrations of 0.06, 0.125, 0.25, 0.50 and 1.0 EU/ml according to the validation of the method. The sample solution, either neat or diluted (expressed as the minimum valid dilution), without exceeding the maximum valid dilution, defined by the endotoxin limits specified in the Pharmacopoeias or calculated on the basis of the threshold human pyrogenic dose of endotoxin and the maximum human dose of the product per kg of body weight (USP 25, 2001), was spiked at a concentration equal to or near the middle of the standard curve. The sample was considered free of interfering factors if the measured concentration of the endotoxin added to the sample solution was within 50–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution to which endotoxin had not been added.

3. Results

3.1. Human peripheral blood monocytes assay (PBMNC)

A typical standard curve from the ELISA of the First International Standard for interleukin-6 was constructed with concentrations in triplicate from 3.9 to 2000 pg/ml, obtaining $R^2 = 0.9966$, and the standard curve of the Second International Standard for bacterial endotoxin at concentrations from 0.06 to 1 EU/ml giving an $R^2 = 0.9974$, by the PBMNC (data not shown).

The ELISA of IL-6 was used to quantify endotoxin-evoked immunoreactive IL-6 production by monocytes from human peripheral blood. Concentrations of IL-6 secreted by cells incubated for 16–24 h with *E. coli* endotoxin are shown in Fig. 1.

The detection limit for bacterial endotoxins was 0.06 EU/ml established in 10 independent assays, each one with three standard curves, with a mean value of 0.06 ± 0.015 EU/ml.

3.1.1. Interference

Three independent assays were performed for all the parenteral pharmaceutical products and the results, presented in Table 1, show that all the samples were free of interference. The spike recovery from the lower valid dilution after spiking with 0.250 EU/ml of the Second International Standard for bacterial endotoxin was between 62 and 165%.

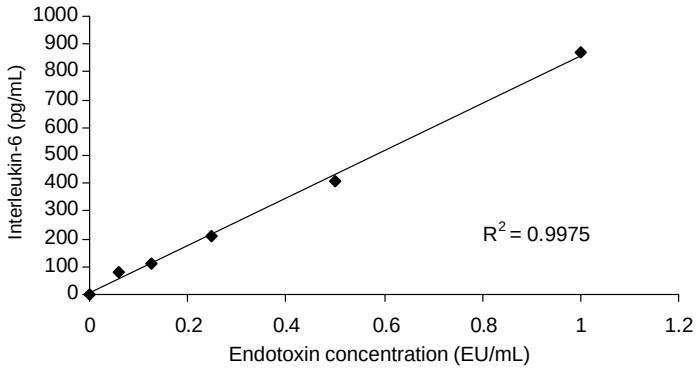

Fig. 1. The dose–response curves for immunoreactive IL-6 secretion by human peripheral blood monocytes stimulated with the Second International Standard of bacterial endotoxin, where $R^2 = 0.9975$ and $y = 854.01x + 4.4667$.

The comparative results of the PBMNC test, the LAL test and the in vivo rabbit pyrogen test are shown in Table 2. The data show the different detection limits and the minimum valid dilution interference-free for the PBMNC, always with concentrations, in EU/ml, lower than that of the maximum valid dilution accepted. It should be noted that the products identified as ampicillin A, B and erythropoietin C were initially tested for LAL-reactive β-glucan, giving the values 153.6–307.2, 30.72–61.44 and 1.92–3.84 EU/ml, respectively, showing the interference of the reagent. Table 2 shows the LAL-non-reactive β-glucan results. The values are expressed in EU/ml by the relationship between the minimum valid dilution without interference and the sensitivity of the PBMNC.

3.2. Human whole blood cell culture assay (WBC)

The standard curve for the Second International Standard of bacterial endotoxin was generated with the concentrations of 0, 0.03, 0.06, 0.125, 0.25, 0.50, 1, 2, 4 and 8 EU/ml, showing a linear region between 0.06 and 1 EU/ml with $R^2 = 0.9925$ and a sensitivity of 0.06 EU/ml.

Table 1
Results of the interference test of pharmaceutical products by the human peripheral blood monocytes assay (PBMNC)

Product	Endotoxin limit	Maximum valid dilution	Minimum valid dilution	Spike recovery[a] (%)
Human serum albumin 20%	0.025 EU/mg[b]	1:83	1:80	122 ± 17
Ampicillin 1000 mg/5 ml	0.15 EU/mg	1:500	1:100	158 ± 18
Enoxaparin 100 mg/ml	0.01 EU/U anti-Xa	1:1666	1:20	140 ± 10
Erythropoietin 4000 IU/vial	5 EU/4000 Iu[b]	1:83	1:20	152 ± 15
Erythropoietin 2000 IU/vial	2.5 EU/2000 IU[b]	1:42	1:20	147 ± 12
recG-CSF 300 mcg/vial	2 EU/ml[b]	1:33	1:10	155 ± 26
Calcium folinate 50 mg/5 ml	0.6 EU/mg[b]	1:100	1:40	98 ± 17
Gentamicin 80 mg/2 ml	1.7 EU/mg	1:1133	1:50	62 ± 8
Insulin 100 U/ml	80 EU/100 U	1:1333	1:50	158 ± 29
Metoclopramide 10 mg/2 ml	2.5 EU/mg	1:208	1:50	65 ± 14
Oxacilin 500 mg/5 ml	0.2 EU/mg	1:333	1:50	120 ± 5
Pantoprazol 40 mg/10 ml	8.75 EU/mg[b]	1:583	1:50	99 ± 12
Ranitidine 25 mg/ml	7.0 EU/mg	1:2917	1:100	156 ± 25
Saline solution 0.9%	0.5 EU/ml	1:8.4	1:5	94 ± 7
Tenoxican 40 mg/2 ml	8.75 EU/mg[b]	1:2916	1:100	165 ± 15

[a] Mean ± S.D. ($n = 3$).
[b] Calculated.

Table 2
Comparative results of pyrogens evaluation in pharmaceutical products by the human peripheral blood monocytes assay (PBMNC), the *Limulus* amoebocyte lysate (LAL) test and the rabbit pyrogen test

Product	Number of batches	PBMNC (EU/ml)	LAL (EU/ml)	Rabbit test
Ampicillin 1000 mg/5 ml A	1	<6	<0.06	Pass
Ampicillin 1000 mg/5 ml B	1	<6	<0.06	Pass
Gentamicin 80 mg/2 ml	2	<3	<0.06	Pass
Oxacilin 500 mg/5 ml	2	<3	<0.06	Pass
Enoxaparin 100 mg/ml	3	<1.2	<0.06	Pass
Insulin 100 U/ml	2	<3	<0.06	Pass
Tenoxican 40 mg/2 ml	1	<6	<0.06	Pass
Metoclopramide 10 mg/2 ml	4	<3	<0.06	Pass
Calcium folinate 50 mg/5 ml	1	<2.4	<0.06	Pass
Ranitidine 25 mg/ml	2	<6	1.2–2.4	Pass
Pantoprazol 40 mg/10 ml	1	<3	<0.06	Pass
Human serum albumin 20%	1	<4.8	0.48–0.96	Pass
Erythropoietin 4000 IU/vial A	1	<1.2	0.48–0.96	Pass
Erythropoietin 2000 IU/vial B	1	112 ± 10[a]	491.5–983	Fail
Erythropoietin 4000 IU/vial C	1	<1.2	<0.06	Pass
recG-CSF 300 mcg/vial A	3	<0.6	<0.06	Pass
Saline solution 0.9% A	1	<0.3	<0.06	Pass

[a] Mean ± S.D. ($n = 3$).

The ELISA of IL-6 was used to quantify endotoxin-evoked immunoreactive IL-6 secretion by the WBC. The effects of variation between donors were also investigated and it can be seen from Fig. 2 that, although there was great variation between the amounts of IL-6 secreted by different individuals, there is a linear relationship between IL-6 secretion and the amount of endotoxin added to the culture for endotoxin concentrations above 0.06 EU/ml. These results indicate that the slope of the curve is an inherent property of the cells from an individual donor to synthesise and release IL-6. Due to the variation of slopes for endotoxin-stimulated IL-6 secretion, an endotoxin standard curve must be included for each experiment and the concentration of pyrogens is expressed in relation to the standard.

A comparison of the sensitivity to specific pyrogenic sources was carried out by incubating the blood with the dilutions of the inactivated Gram-negative bacterium *E. coli*, the fungus *C. albicans* and the

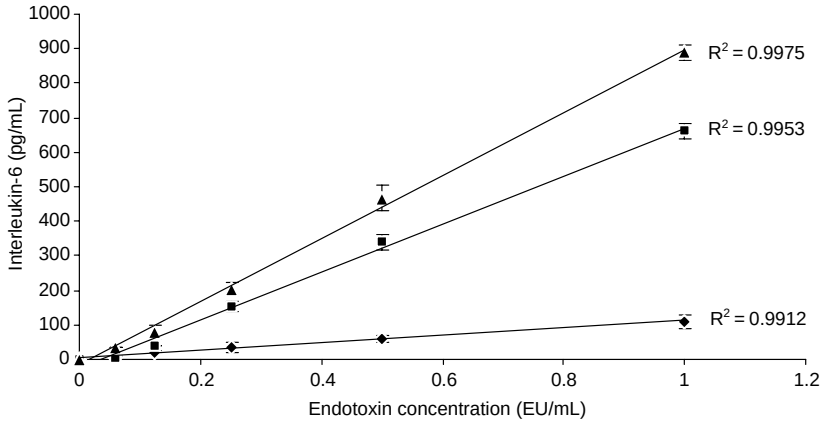

Fig. 2. Interleukin-6 secretion by human whole blood cell cultures from three different donors (▲, ■, ◆) upon stimulation with the Second International Standard of bacterial endotoxin. Data ($n = 4$) are mean ± S.D.

Gram-positive bacterium *S. aureus*, which contain pyrogenic components other than endotoxin. Fig. 3 shows the sensitivity in each case and the linearity between the concentration and quantity of IL-6 released with the standard deviation, demonstrating the efficiency of the assay for the detection of these possible pyrogen contaminants. The dilution of 25,000 cfu/ml gave levels of IL-6 secretion of 112 pg/ml for *E. coli*, 33.55 pg/ml for *C. albicans* and 50.77 pg/ml for *S. aureus*, that are comparable to the mean responses ($n = 3$) obtained with 0.16 ± 0.02, 0.07 ± 0.014 and 0.09 ± 0.02 EU/ml, respectively of the Second International Standard of Bacterial endotoxin.

3.2.1. Interference

The interference test was carried out giving spiked recovery values of the bacterial endotoxins, added to the lower valid dilution, within 58 and 132% (Table 3), demonstrating the absence of interference.

The parenteral pharmaceutical products were assessed by the WBC assay, and comparing this with the rabbit pyrogen test and the LAL test (Table 4). Dexamethasone was also tested with concentrations within 0.4 and 0.002 mg/ml, although it gave no response, probably because it inhibits/abolishes the production of IL-6.

The WBC assay was also used for the evaluation of pharmaceutical products spiked with 25 EU/ml of the Second International Standard of bacterial endotoxin. This dose is nearly double the 13.81 EU/ml per kg of body weight that was previously shown experimentally to induce a positive response in rabbits with an individual increase in temperature of 0.5 °C (data not shown). The results for three independent donors show significant reproducibility and a calculated spike recovery (Table 5) of between 82 and 117%.

Table 3
Results of the interference test of pharmaceutical products by the human whole blood cell culture assay (WBC)

Product	Endotoxin limit	Maximum valid dilution	Minimum valid dilution	Spike recovery[a] (%)
Human serum albumin 20%	0.025 EU/mg[b]	1:83	1:40	88 ± 11
Amikacin 500 mg/2 ml	0.33 EU/mg	1:1375	1:200	90 ± 18.5
Ampicillin 1000 mg/5 ml	0.15 EU/mg	1:500	1:100	70 ± 10
Ketoprofen 100 mg/2 ml	3.5 EU/mg[b]	1:2917	1:100	72 ± 7.5
Cytarabine 100 mg/5 ml	0.07 EU/mg	1:23	1:20	63 ± 16
Diclofenac 75 mg/3 ml	4.7 EU/mg[b]	1:1958	1:200	76 ± 9.0
Dipyrone 500 mg/ml	0.35 EU/mg[b]	1:2917	1:400	96 ± 10
Enoxaparin 100 mg/1 ml	0.01 EU/U anti-Xa	1:1666	1:10	94 ± 26
Erythropoietin 4000 IU/vial	5 EU/4000 IU[b]	1:83	1:10	88 ± 5.0
recG-CSF 300 mcg/vial	2 EU/ml[b]	1:33	1:10	93 ± 2.5
Calcium folinate 50 mg/5 ml	0.6 EU/ml[b]	1:100	1:10	132 ± 22
Furosemide 10 mg/ml	3.6 EU/mg	1:600	1:10	58 ± 8.0
Gentamicin 80 mg/2 ml	1.7 EU/mg	1:1133	1:100	77 ± 5.0
Heparin 5000 IU/ml	0.03 EU/U	1:2500	1:10	70 ± 20
rec-hGH 4 IU/vial	5 EU/mg	1:133	1:20	94 ± 2.0
Insulin 100 U/ml	80 EU/100U	1:1333	1:100	98 ± 7.0
Metoclopramide 10 mg/2 ml	2.5 EU/mg	1:208	1:50	104 ± 19
Oxacilin 500 mg/5 ml	0.2 EU/mg	1:333	1:100	90 ± 21
Pantoprazol 40 mg/10 ml	8.75 EU/mg[b]	1:583	1:100	95 ± 1.5
Ranitidine 25 mg/ml	7.0 EU/mg	1:2917	1:100	64 ± 8.5
Saline solution 0.9%	0.5 EU/ml	1:8.4	1:5	94 ± 2
Glucose 0.5%	0.5 EU/ml	1:8.4	1:5	93 ± 2.5
Tenoxican 40 mg/2 ml	8.75 EU/mg[b]	1:2916	1:100	82 ± 10
Vancomycin 500 mg/5 ml	0.33 EU/ml	1:550	1:100	124 ± 19
Vitamin K 10 mg/ml	17 EU/ml	1:2333	1:100	75 ± 1

[a] Mean ± S.D. ($n = 3$).
[b] Calculated.

Table 4
Comparative evaluation results of pharmaceutical products from the human whole blood cell culture assay (WBC), *Limulus* amoebocyte lysate test (LAL) and the rabbit pyrogen test

Products	Number of batches	WBC (EU/ml)	LAL (EU/ml)	Rabbit test
Dipyrone 500 mg/ml	3	<24	<0.06	Pass
Amikacin 500 mg/2 ml	2	<12	<0.06	Pass
Ampicillin 1000 mg/5 ml A	1	<6	<0.06	Pass
Ampicillin 1000 mg/5 ml B	1	<6	<0.06	Pass
Gentamicin 80 mg/2 ml	2	<6	<0.06	Pass
Oxacilin 500 mg/5 ml	2	<6	<0.06	Pass
Vancomycin 500 mg/5 ml	2	<6	<0.06	Pass
Enoxaparin 100 mg/ml	3	<0.6	<0.06	Pass
Heparin 5000 IU/ml	2	<0.6	<0.06	Pass
Insulin 100 U/ml	3	<6	<0.06	Pass
Ketoprofen 100 mg/2 ml	1	<6	<0.06	Pass
Diclofenac 75 mg/3 ml	1	<12	<0.06	Pass
Tenoxican 40 mg/2 ml	2	<6	<0.06	Pass
Metoclopramide 10 mg/2 ml	3	<3	<0.06	Pass
Cytarabine 100 mg/5 ml	1	<1.2	<0.06	Pass
Calcium folinate 50 mg/5 ml	1	<0.6	<0.06	Pass
Ranitidine 25 mg/ml	1	<6	1.2–2.4	Pass
Pantoprazol 40 mg/10 ml	1	<6	<0.06	Pass
Furosemide 10 mg/ml	2	<0.6	<0.06	Pass
rec-hGH 4 IU/vial A	2	<1.2	<0.06	Pass
rec-hGH 4 IU/vial B	1	12.4 ± 2.5[a]	15.84–31.68	Pass
Human serum albumin 20%	1	<2.4	0.48–0.96	Pass
Erythropoietin 4000 IU/vial A	1	0.76	0.48–0.96	Pass
Erythropoietin 2000 IU/vial B	1	141 ± 2.8[a]	491.5–983	Fail
Erythropoietin 4000 IU/vial C	1	<0.6	<0.06	Pass
recG-CSF 300 mcg/vial	3	<0.6	<0.06	Pass
Saline solution 0.9% A	2	<0.3	<0.06	Pass
Saline solution 0.9% B	1	44.8 ± 5[a]	48–96	Fail
Glucose 0.5%	1	2054 ± 95[a]	1920–3 840	Fail
Vitamin K 10 mg/ml	2	<6	<0.06	Pass

[a] Mean ± S.D. ($n = 3$).

Table 5
Human whole blood cell culture assay (WBC) spiked recovery of parenteral pharmaceutical products with 25 EU/ml of the Second International Standard for bacterial endotoxin

Products	Endotoxin added (EU/ml)	Spike recovery (EU/ml)[a]
Erythropoietin 4000 IU/vial	25	23.3 ± 4.0
recG-CSF 300 mcg/vial	25	22.1 ± 2.0
Calcium folinate 50 mg/5 ml	25	29.3 ± 6.0
Furosemide 10 mg/ml	25	21.0 ± 1.6
Heparin 5000 IU/ml	25	27.7 ± 2.1
rec-hGH 4 IU/vial	25	20.5 ± 2.3
Saline solution 0.9%	25	24.2 ± 1.8
Vancomycin 500 mg	25	29.2 ± 5.6

[a] Mean ± S.D. ($n = 3$).

4. Discussion

Among the methodologies investigated as in vitro alternatives for the assay of pyrogens (Taktak et al., 1991; Eperon and Jungi, 1996; Moesby et al., 1999) the human whole blood cell culture (WBC) (Hartung and Wendel, 1996; Pool et al., 1998) and the human PBMNC (Hansen and Christensen, 1990) tests were chosen for further study considering the importance and necessity of these alternatives for the quality control of parenteral preparations.

With regard to the choice of readout (measured variable), immunoreactive IL-6 was considered the most appropriate because this is secreted entirely into the

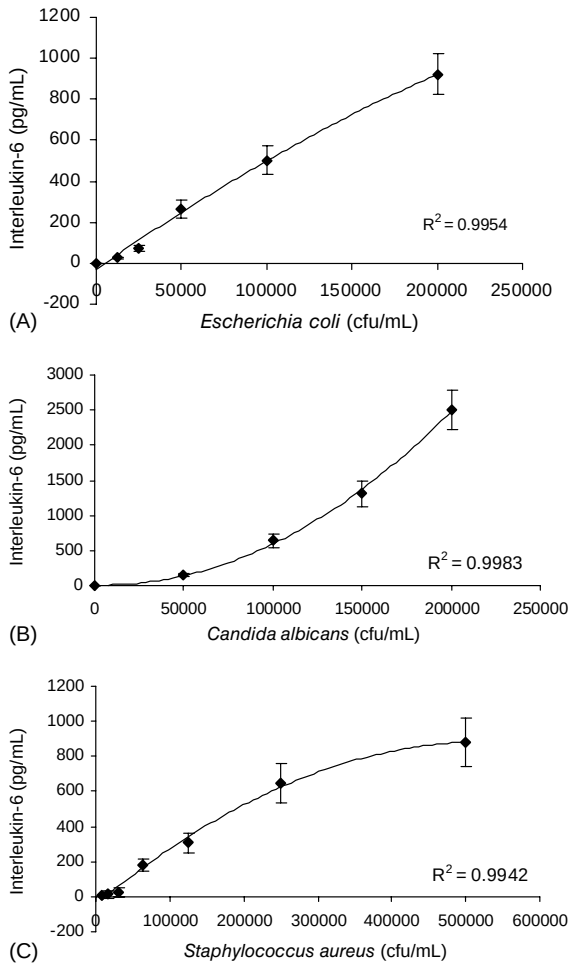

Fig. 3. Interleukin-6 secretion in human blood cell culture incubated in the presence of (A) *E. coli*, (B) *C. albicans* and (C) *S. aureus*. Data ($n = 4$) are mean ± S.D.

blood/medium, in large quantities, permitting its accurate estimation (Poole et al., 1989; Taktak et al., 1991; Moesby et al., 1999; Poole and Gaines Das, 2001). Also, a recent study by Nakagawa from the Japanese NIH compared TNFα, IL-1β and IL-6 as readouts, with diluted whole blood and a monocytic cell line as the cellular sources, and reported that the structurally diverse pyrogens endotoxin, peptidoglycan, *S. aureus* Cowan 1 and poly(IC) all stimulated the release of more IL-6 than either TNFα or IL-1β. More importantly, IL-6 was induced by lower concentrations of each pyrogen than were required to induce TNFα and IL-1β (Nakagawa et al., 2002).

The PBMNC test was pre-validated and the interference test (Table 1) satisfied the specifications with spike recovery limits between 50 and 200% (Jahnke et al., 2000; Poole and Gaines Das, 2001) and, since the samples are known to be interference-free, this test is not necessary in subsequent analysis.

Pharmaceutical products were assayed by the PBMNC, *Limulus* amoebocyte lysate and rabbit pyrogen tests (Table 2). The methodologies under investigation gave comparable results (though with approximately 100 times lower sensitivity) to the LAL test and, with certain products, such as with the sample of recombinant human erythropoietin B, they were more robust than the LAL test, being less prone to inhibition by interfering factors.

Compared to the other tests, the PBMNC test requires more sophisticated techniques and expensive reagents. In general, the PBMNC/IL-6 test is less prone to product interference than the WBC/IL-6, permitting less dilute drugs to be tested. This is very important because non-endotoxin pyrogens dilute out more rapidly than endotoxin, so the WBC for pyrogens/LPS cytokines induction was evaluated in parallel with the PBMNC test. This followed consideration of the practical advantages including absence of the need for cell separation and, in addition, the fact that the monocytes/leukocytes are cultured in the presence of all the blood components with which they are normally found in vivo (Hartung and Wendel, 1996; Pool et al., 1998). It is relevant to point out the use in both methods of freshly isolated human blood, that is associated with some shortcomings related to the risk of infection, as well as ethical concerns, but this is justified by the necessity for the development of new alternatives to the existing assays for the evaluation of pyrogens contamination of pharmaceutical products.

Variations in the individual responses were addressed by examining in parallel, in the same culture plate, the Second International Standard of bacterial endotoxin. This was the logical choice as the standard considering the observations that Gram-negative bacteria are the most frequent contaminants found in pharmaceutical products (Jahnke et al., 2000; Poole and Gaines Das, 2001).

The pre-validation of the WBC assay was confirmed by the linearity of the dose–response curve and the interfering factors test suggested for this methodology (Hartung and Wendel, 1996; Jahnke et al., 2000). The

spike recovery from the lower dilution without interference was between 50 and 200%, and satisfied the criteria for freedom from interference (Jahnke et al., 2000; Poole and Gaines Das, 2001).

The pharmaceutical products exhibited consistency between the WBC assay, the LAL and the rabbit pyrogen test, considering the specifications for the endotoxin limits, although the quantitative results were more precise and reproducible (Table 4). Reproducibility was also evaluated with the spiked samples (Table 5), which presented a mean recovery value of 98.8%.

The WBC assay was evaluated in more detail since, similar to the PBMNC, it can accurately detect the pyrogenic status of the pharmaceutical products. The former has advantages over the rabbit assay in that it is more sensitive and, unlike the LAL assay, it can detect pyrogens other than endotoxin from Gram-negative bacteria as demonstrated in the experiment with Gram-positive bacteria (Fig. 3), despite being a less potent inducer of IL-6 than the Gram-negative bacteria. The two in vitro tests for pyrogens exhibited good agreement overall, both with each other and with the LAL test and the rabbit pyrogen test for the detection of the endotoxins that are the pyrogens that usually contaminate drugs. Additional work needs to be performed to evaluate the two assay systems for the detection of non-endotoxin pyrogens. The experiments reported here on the application of the in vitro standardised and validated assays to a broader class of medicinal products extend the data in the literature and provide important new results, which represent a contribution towards the application of these tests to improving the quality control of parenteral preparations.

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