Monocyte Activation Test (MAT) Reliably Detects Pyrogens in Parenteral Formulations of Human Serum Albumin

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Summary

Disadvantages of the regulatory pyrogen test to assure safety of the end-product Human Serum Albumin (HSA) for parenteral use call for the implementation of an alternative test. In the current study, 16 HSA batches were assayed for pyrogens in parallel with the Rabbit Pyrogen Test, conventional and endotoxin-specific LAL assay and monocyte activation test (MAT).

It was found that all HSA batches were contaminated with (1,3)-β-glucans, which interfere with the conventional LAL. Endotoxin-specific LAL was not suitable to test HSA due to unacceptable endotoxin recovery. Experiments combining polymyxin B and MAT demonstrated that pyrogenic batches were mainly contaminated with endotoxins. However, endotoxin-specific LAL failed to detect one of them. The contaminating (1,3)-β-glucans enhanced the MAT/IL-6 response to endotoxin, but not that of MAT/IL-1β. The endotoxin equivalent concentrations obtained using the IL-6 readout were usually higher than those using IL-1β, probably owing to the direct induction of IL-6 release from monocytes by (1,3)-β-glucans.

The MAT correlates with the rabbit pyrogen test, providing a higher safety level for pyrogenicity testing of HSA and probably other therapeutic proteins.

Keywords: pyrogen, albumin, (1,3)-β-glucans, endotoxin, monocyte activation test

1 Introduction

Pyrogens are fever-inducing substances usually derived from microorganisms that could induce deleterious responses to the host, including septic shock or even death, when entering the blood stream (Dinarello, 2004). Therefore, manufacturers of pharmaceutical parenteral products and medical devices that will be in contact with the systemic circulation are responsible for ensuring that their products are non-pyrogenic.

There are now three official pharmacopoeial tests to detect pyrogens in parenteral products: the Rabbit Pyrogen Test (RPT), the bacterial endotoxin test (BET), often referred to as the Limulus Amebocyte Lysate test (LAL), as well as the Monocyte Activation Test (MAT), of which there are five variants.

Due to the high consumption of animals required by RPT and its ethical implications, this test has been replaced by LAL in most pharmacopoeial monographs. However, most regulators continue to recommend the RPT for the evaluation of plasma-derived medicinal products.

The MAT is based on the *in vitro* activation of human monocytoid cells by pyrogens, which leads to the release of proin-flammatory cytokines that are determined by ELISA (Hartung et al., 2001). Five variants of the MAT have recently been standardized and validated (Hoffmann et al., 2005). They have all been accepted by the European Centre for the Validation of Alternative Methods (ECVAM) as alternatives to the RPT (ESAC Statement, 2006) and were adopted by the European Pharmacopoeia in 2010 (EP, 2010).

Nevertheless, according to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the MAT cannot be considered a complete replacement of the RPT, due mainly to the lack of comparative data between the two methods. However, it can be employed as a substitute for detecting Gram-negative endotoxin in parenteral drugs on a case-by-case basis (ICCVAM, 2008, 2009). The same position has been adopted by the US Food and Drug Administration (FDA) (FDA, 2009).

Blood-derived product manufacturing presents specific challenges in relation to microbiological contamination, due to the possibility of initial contamination of plasma units and to the risk of introducing bacterial contaminations during the subsequent production steps. Moreover, as the main fever induc-

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ing cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα) are natural constituents of human plasma, they can also be found in plasma pools for fractionation (EMEA, 2009). The European Medicines Agency (EMEA) encourages the replacement of RPT by alternative tests such as LAL or MAT in plasma-derived medicinal products (EMEA, 2009).

Human serum albumin (HSA) is an important plasma-derived medicinal product that has been used for more than 60 years as a parenteral therapeutic agent for the maintenance and restoration of blood volume (Mc Clelland et al., 1990). There are several reports on HSA products that have passed RPT and LAL but have elicited pyrogenic episodes in patients (Pool and McLeod, 1995; Poole et al., 1988a,b; Steere et al., 1978; Taktak et al., 1991), indicating that these tests do not always provide sufficient safety support.

Therefore, an improved safety testing strategy for pyrogens is required for HSA. Previous comparative studies between MAT and RPT on HSA showed that the whole blood MAT variant (WB-MAT) resulted in the same level of safety as the RPT (Spreitzer et al., 2002). Also, the MAT was more effective in detecting endotoxin at the borderline concentration, which often causes the RPT to fail, resulting in further assays with additional animals. Others have also found that MAT is a promising test system for pyrogenic contaminations of HSA (Poole et al., 1988a,b; Taktak et al., 1991).

Here we have carried out a parallel comparison of RPT, LAL, and MAT as end-product pyrogen test for HSA preparations. Samples of the 16 batches were not modified or artificially spiked with pyrogens, providing a real-life picture of the performance of each test. The MAT demonstrated the highest safety level for detection of pyrogens in HSA.

2 Animals, materials, and methods

Samples and reagents
In this study, sixteen batches of end-product 20% HSA for parenteral use (referred to as batch A to P) were assayed for pyrogens by the methods described below. The 2nd International Standard for Endotoxin (NIBSC code: 94/580, UK) (Poole et al., 1997), reconstituted and stored as recommended in the manufacturer's instructions, was used as standard in MAT as described elsewhere (Andrade et al., 2003). The International Standard of human IL-1β (rDNA derived, NIBSC code: 86/552, UK) (Poole and Gaines-Das, 1991), and WHO 1st International Standard of human IL-6 (NIBSC code: 89/548, UK) (Gaines-Das and Poole, 1993), were reconstituted in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) and stored at -80°C until use. Dilutions of cytokine standards for ELISA were done in the same buffer. All consumables were purchased sterile and certified pyrogen-free. Glassware was depyrogenated by dry-heat at 250°C for 1 h.

Determination of endotoxin limit concentration (ELC)
The endotoxin limit concentration was calculated as ELC = K/M, with K being the threshold pyrogenic dose of endotoxin per kilogram of body mass per day (120 EU/kg/24 h) and M the daily maximum recommended bolus dose of product per kg body mass (FDA, 1987). Maximum dose of HSA according to the Cuban national formulary of pharmaceuticals is 6 g/kg/24 h (FNM, 2006), which is equivalent to 30 ml/kg/24 h for 20% HSA. Therefore, an endotoxin limit of 4 EU/ml was used throughout this study as pass/fail criterion for LAL and MAT.

Rabbit pyrogen test
RPT in 20% HSA was carried out according to the monograph described in the European Pharmacopoeia (EP, 2007) by injecting 5 ml per kg of body weight.

Limulus amebocyte lysate assay
Determination of endotoxin by LAL was conducted by the kinetic chromogenic method using the Pyrochrome® kit (Associates of Cape Cod, E. Falmouth, MA, USA), according to the manufacturer's instructions. The reaction was kinetically measured at 405 nm and 37°C for one hour with a microplate reader Biotek Elx808™ IU (BioTek® Instruments, Winooski, VT, USA). The data was processed with KC4™ v.3.4 (BioTek® Instruments, Winooski, VT, USA). The level of interference in the samples and its dilutions was assessed using endotoxin spikes at 0.5 EU/ml. Where indicated, LAL reagent was reconstituted with Glucashield® buffer (Associates of Cape Cod), producing an endotoxin-specific LAL reagent (ES-LAL). HSA samples were diluted in borosilicate tubes (Associates of Cape Cod) to avoid endotoxin adsorption to polystyrene tubes promoted by albumin as described by Yokota et al. (2001). The maximum valid dilution (MVD) for HSA was 1:400.

Monocyte activation test using fresh human whole blood (WB-MAT)
a) Human whole blood incubation
Blood from healthy volunteers was drawn using syringes (Multifly®, Sarstedt, Nümbrecht, Germany) connected to sodium heparin coated tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany). Human whole blood incubations were performed as described elsewhere (Daneshian et al., 2009; Spreitzer et al., 2002). In brief, 100 μl of two-fold serial dilutions of HSA in saline were mixed with 1 ml saline and 100 μl fresh human blood in 1.5 ml polypropylene reaction tubes (Eppendorf, Hamburg, Germany). The tubes were shaken gently by inversion and incubated overnight (16-24 h) at 37°C. Thereafter, samples were shaken again and centrifuged for 5 min at 13,000 rpm. Supernatants were stored at -80°C until cytokine measurements by ELISA were performed.

b) Enzyme-linked immunosorbent assay (ELISA)
Cytokine measurements were carried out with an in-house sandwich ELISA, based on matched antibodies against human IL-1β or IL-6 (Pierce Biotechnology, Rockford, IL, USA) diluted with PBS containing 3% BSA. 50 μl of 2 μg/ml coating monoclonal anti-IL-1β or 1 μg/ml coating monoclonal anti-IL-6 was added to each well of a microtiter plate (F96 Nunc-Immuno Plate, Polysorp, Nunc, Roskilde, Denmark) and incubated for 16-24 h at 4°C. The plates were coated with 200 μl blocking

buffer (PBS containing 3% BSA) for 2 h at 25 °C, and washed three times with PBS containing 0.5% Tween 20 (Sigma, St. Louis, MO, USA). Supernatant (50 μl) from the whole blood incubation and 50 μl of biotin-labeled monoclonal antibody (0.25 μg/ml anti-IL-1β or 0.5 μg/ml anti-IL-6) were added per well and further incubated for 2 h at 25°C. After washing four times, 100 μl of streptavidin-peroxidase (Sigma, St. Louis, MO) were added into each well and incubated for 30 min. Then plates were washed eight times and 100 μl of 3,3´,5,5´tetramethylbenzidine (TMB) (Sigma, St. Louis, MO, USA) was added. After 10-15 min the reaction was stopped with 50 μl 1 M H$_2$SO$_4$. The absorbance was measured at 450 nm with 630 nm as reference wavelength in a microplate reader Biotek Elx808™ IU (BioTek® Instruments, Winooski, VT). The MAT response to endotoxin was assessed from the dose-response plot of endotoxin standard concentration (from 0.03125 to 2 EU/ml) vs. absorbance. As MAT can detect pyrogens other than endotoxins, results were presented as endotoxin equivalent units per ml (EEU/ml) as suggested by Montag et al. (2007).

c) Test for interferences
Demonstrating the absence of interfering factors is required to guarantee reliable results of the assay. The test was conducted by incubating diluted whole blood with sample dilutions spiked with 0.5 EU/ml and assayed in parallel with the corresponding unspiked dilution. Dilutions with endotoxin recovery within the 50-200% range were considered interference-free, and the first was defined as minimum valid dilution (MinVD).

d) Determination of IL-1β and IL-6 concentrations
Where indicated, cytokine concentrations of undiluted HSA samples were determined by the ELISA procedure described above, along with the corresponding cytokine standard curve ranging from 6 to 500 pg/ml for IL-1β and from 1 to 200 pg/ml for IL-6. Cytokine concentrations were obtained by linear regression of the linear portion of the curve of cytokine concentration vs. absorbance.

e) Differentiation between endotoxin and non-endotoxin pyrogen contamination
HSA samples that failed RPT were incubated with Polymyxin B sulphate (PMB) (Sigma, St. Louis, MO) to elucidate whether they were contaminated with endotoxin or not (Pool et al., 1999). The sample diluted in its MinVD was incubated with whole blood and 1 mg/ml PMB (final concentration in the tube) (Huszar et al., 2002). Negative control treated with PMB was employed as assay control. IL-1β was measured by ELISA. Statistical significance was determined by using one-way ANOVA with GraphPad Prism 5.0 (GraphPad software, San Diego, CA).

3 Results

Rabbit pyrogen test
Batches G, H and I failed the RPT according to the European Pharmacopoeia guidelines (see the resumed comparative study in Tab. 3), i.e. these batches showed pyrogenic contamination.

LAL assay
It is well established that a valid LAL test requires the absence of interference (inhibition or enhancement) in the endotoxin activity, which is determined by the level of endotoxin recovery from positive product controls (spike) (FDA, 1987). The LAL method used in the current study requires an endotoxin recovery between 50-200%. Typical results of the interference assay using conventional LAL in 10 batches are depicted in Table 1. In samples from batches B, G, I, J and K we found that endotoxin recovered from the spikes exceeded the upper limit. When samples were diluted, the over-response decreased until the endotoxin recovery fell within the accepted range. In addition to endotoxin, the only known molecules able to activate the Limulus cascade are (1,3)-β-glucan containing structures, which are able to produce false positive results for endotoxin contamination (Cooper et al., 1997). Since it has been previously demonstrated that glucans enhance the LAL response to endotoxins, and that this effect decreases with sample dilution (Roslansky and Novitsky, 1991), we suspected the observed behavior was likely due to (1,3)-β-glucan contamination.

Therefore, HSA samples were also assayed with an endotoxin-specific LAL reagent (ES-LAL). The enhancing effect was abolished and the final endotoxin concentrations were significantly lower than those obtained by C-LAL, except for batch H (Tab. 1), thus demonstrating the presence of (1,3)-β-glucans.

For five batches we found an out of range spike recovery at all dilutions. For that reason, no valid dilution could be stated for the validation/interference assay (Tab. 1). Hence, ES-LAL is not a suitable endotoxin test for HSA.

Interestingly, the spike was recovered at all dilutions in batches G, H and I, which had failed RPT. However, only batches H and I failed the ES-LAL (Tab. 3).

In general, 1:100 dilutions or higher produced concentrations below the lowest endotoxin concentration of the standard curve (0.01 EU/ml) when using the ES-LAL method. Consequently, the results obtained at 1:10 dilutions are only displayed in the comparative analysis shown in Table 3.

Monocyte activation test (MAT)
We first determined the linear quantification range under our assay conditions. A linear relationship was found from 0.125 to 1 EU/ml for IL-1β and from 0.0625 to 1 EU/ml for IL-6 (plots not shown), which is in accordance with previous results (Andrade et al., 2003; Poole et al., 2003). The correlation coefficient for both curves was typically greater than 0.99. MVD were 1:32 and 1:64 for IL-1-β and IL-6 readouts, respectively.

The interference test for HSA in WB-MAT was conducted three times in clean samples of five different batches. As the quantification range in MAT was up to 1 EU/ml LPS with both cytokine readouts, and the spikes were 0.5 EU/ml, it was only possible to quantify an enhancing effect up to 200%. Here we found an enhanced IL-6 response to LPS in the less diluted samples, which was abrogated with sample dilution. This enhanced response was not evidenced with IL-1β (Tab. 2).

The MinVDs for 20% HSA were 1:32 and 1:4 for IL-6 and IL-1β readout, respectively, and these dilutions were chosen for the assay. Batches G and H clearly failed MAT with both

Tab. 1: Interference test of 20% HSA in Conventional and Endotoxin-Specific LAL assays

Batch	Dilution	C-LAL		Dilution	ES-LAL	
		Concentration (EU/ml)[a]	Spike recovery (%)		Concentration (EU/ml)[a]	Spike recovery (%)
A	10	23.46 ± 0.709	52.2	10	0.05 ± 0.006	52.4
	100	0.51± 0.021	158.0	100	< 0.01	39.4
	300	0.16 ± 0.002	170.2	400	< 0.01	41.8
	500	0.12 ± 0.009	121.4			
B	10	2.33 ± 0.069	230.6	10	0.06 ± 0.003	32.6
	100	0.15 ± 0.011	75.1	100	< 0.01	37.2
	300	0.09 ± 0.004	62.7	400	< 0.01	38.4
	500	0.05 ± 0.001	52.4			
D	10	1.27 ± 0.025	147.7	10	0.05 ± 0.002	70.8
	100	0.11 ± 0.004	132.7	100	< 0.01	41.6
	300	0.06 ± 0.004	175.1	400	< 0.01	40.8
	500	0.04 ± 0.003	82.2			
F	10	2.33± 0.105	164.1	10	0.08 ± 0.006	49.0
	100	0.19 ± 0.004	103.0	100	< 0.01	39.7
	300	0.08 ± 0.001	78.0	400	< 0.01	35.3
	500	0.05 ± 0.004	129.4			
G	10	0.63 ± 0.000	206.9	10	0.14 ± 0.000	69.5
	100	0.05 ± 0.004	107.0	100	< 0.01	50.9
	300	0.03 ± 0.001	75.9	400	< 0.01	56.9
	500	0.08 ± 0.003	99.9			
H	10	4.62 ± 0.000	136.8	10	1.74 ± 0.064	55.2
	100	0.25 ± 0.000	99.0	100	0.13 ± 0.012	56.8
	300	0.07 ± 0.004	93.6	400	< 0.01	57.7
	500	0.04 ± 0.001	82.6			
I	10	3.10 ± 0.037	529.0	10	0.41 ± 0.019	77.6
	100	0.13 ± 0.001	109.8	100	0.04 ± 0.01	53.4
	300	0.03 ± 0.001	90.2	400	< 0.01	59.8
	500	0.03 ± 0.001	81.6			
J	10	1.95 ± 0.046	213.0	10	0.11 ± 0.008	41.2
	100	0.09 ± 0.004	81.6	100	< 0.01	40.2
	300	0.04 ± 0.001	57.5	400	< 0.01	37.1
	500	0.02 ± 0.001	81.1			
K	10	3.21 ± 0.002	255.0	10	0.13 ± 0.004	38.8
	100	0.26 ± 0.011	189.8	100	< 0.01	44.0
	300	0.09 ± 0.006	105.0	400	< 0.01	48.3
	500	0.06 ± 0.004	99.9			
P	10	5.30 ± 0.156	100.8	10	0.19 ± 0.006	36.1
	100	0.17 ± 0.002	81.5	100	< 0.01	36.0
	300	0.06 ± 0.001	52.3	400	< 0.01	40.1
	500	0.03 ± 0.001	51.0			

[a] The values are the means of three replicates with S.D.

Tab. 2: Endotoxin recoveries (%) obtained in the interference test with 20% HSA in WB-MAT using IL-1β and IL-6 as readout.

Readout	Batch	Dilutions					
		1/2	1/4	1/8	1/16	1/32	1/64
IL-1β	A	169.8	94.3	133.4	106.4	80.5	76.0
	B	117.8	160.7	158.4	90.9	83.8	92.0
	C	12.2	145.7	149.7	120.9	76.6	58.0
	D	70.0	72.2	98.9	148.1	132.6	109.9
	F	66.8	101.2	96.2	78.9	69.4	75.0
IL-6	A	N.D.	N.D.	> 200	> 200	67.7	112.7
	B	N.D.	N.D.	> 200	> 200	129.0	125.5
	C	N.D.	N.D.	> 200	97.4	38.6	154.0
	D	N.D.	N.D.	> 200	> 200	> 200	185.1
	F	N.D.	N.D.	> 200	> 200	157.9	133.3

Recovery was calculated using the mean value of endotoxin equivalent concentrations (EEU/ml) of the spiked
HSA at 0.5 EU/ml (S) and the un-spiked samples (US) as follows: % Recovery = ((S-US)/0.5) x100.
In samples labeled ND (not determined), it was not possible to quantify the endotoxin recovery since both the spike and unspiked
dilutions produced saturating spectrophotometric signals.

Tab. 3: Comparative results of pyrogen evaluation of HSA by Monocyte Activation Test, Rabbit Pyrogen Test and Endotoxin-Specific-LAL

Batch	MAT (IL-1β) (EEU/ml)[a]	MAT (IL-6) (EEU/ml)[a]	RPT (Σ ΔT) (°C)	ES-LAL (EU/ml)[a]
A	0.81 ± 0.242[P]	< 2[P]	0.60[P]	0.52 ± 0.003[P]
B	0.93 ± 0.224[P]	2.22 ± 0.921[P]	0.05[P]	0.61 ± 0.029[P]
C	1.29 ± 0.245[P]	5.10 ± 0.183[F]	0.10[P]	1.30 ± 0.038[P]
D	1.29 ± 0.406[P]	2.52 ± 0.760[P]	0.25[P]	0.54 ± 0.017[P]
E	1.15 ± 0.364[P]	2.83 ± 0.609[P]	0.35[P]	0.64 ± 0.077[P]
F	1.07 ± 0.517[P]	2.62 ± 1.084[P]	0.35[P]	0.82 ± 0.062[P]
G	>8[F]	8.64 ± 1.28[F]	5.30 (first retest)[F]	1.39 ± 0.000[P]
H	>8[F]	14.67 ± 4.31[F]	5.70 (first retest)[F]	15.35 ± 2.480[F]
I	3.57 ± 0.795[P]	3.85 ± 0.75[P]	7.05 (third retest)[F]	4.07 ± 0.187[F]
J	< 0.5[P]	< 2[P]	0.55[P]	1.09 ± 0.077[P]
K	< 0.5[P]	< 2[P]	0.50[P]	1.33 ± 0.039[P]
L	< 0.5[P]	2.35 ± 0.022[P]	1.85 (first retest)[P]	1.03 ± 0.025[P]
M	1.04 ± 0.157[P]	< 2[P]	0.55[P]	0.96 ± 0.035[P]
N	0.68 ± 0.182[P]	2.36 ± 1.006[P]	0.20[P]	0.72 ± 0.000[P]
O	0.80 ± 0.233[P]	2.72 ± 0.985[P]	0.15[P]	1.43 ± 0.048[P]
P	1.48 ± 0.280[P]	< 2[P]	0.35[P]	1.93 ± 0.063[P]

[a] The values are the means of three replicates with S.D.
[P] Passed the assay
[F] Failed the assay

cytokine readouts and this was in accordance with the RPT results, where these batches failed in the first retest. Batch I failed RPT in the third retest and contained an endotoxin equivalent concentration around the 4 EEU/ml limit as determined with WB-MAT with both cytokine readouts (Tab. 3).

There were noticeably higher endotoxin equivalent concentrations obtained with the IL-6 readout than with IL-1 β, insomuch that batch C would fail the test with the IL-6 readout, whereas it passed MAT/IL-1β and RPT (Tab. 3).

Cytokine concentration
IL-1β is an effective stimulator of IL-6 gene expression and protein translation (Dinarello, 2004), whereas IL-6 has an inhibitory effect on IL-1β production (Fenton, 1992; Watkins et al., 1999). As IL-1β and/or IL-6 are conceivable contaminants of blood-derived products, the concentrations of both cytokines were determined in undiluted HSA from batch C.

A linear relationship between IL-1β and IL-6 vs. OD was typically found between 7-500 pg/ml and 1-200 pg/ml, respective-
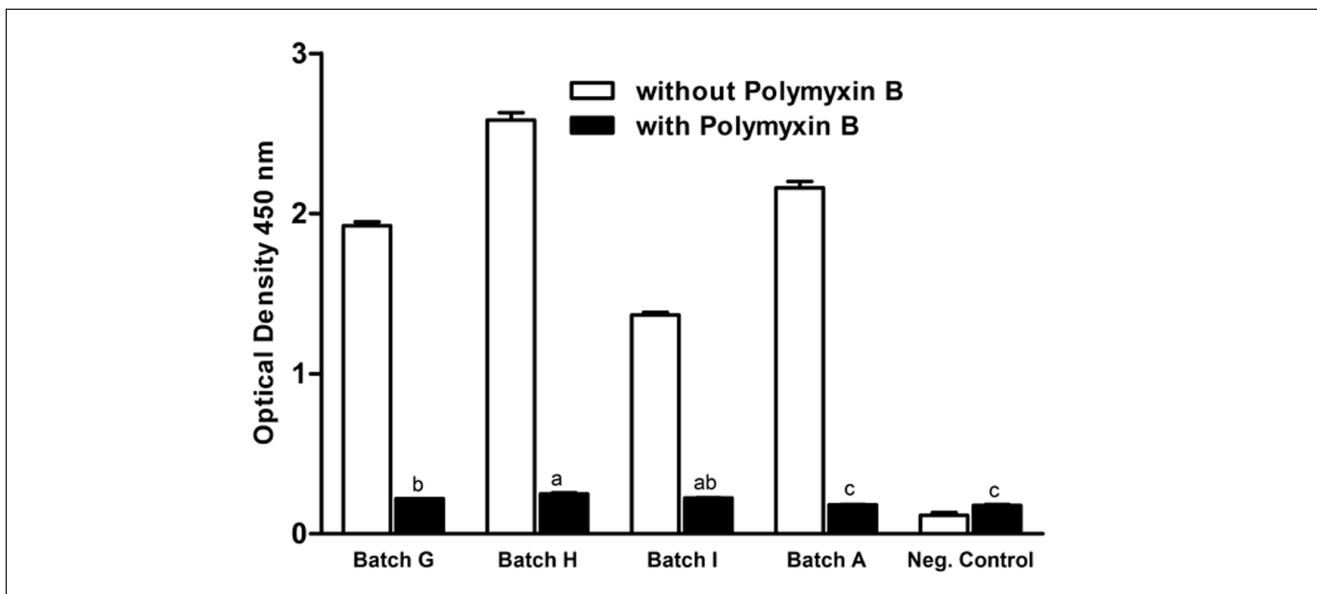
Fig. 1: Effect of polymyxin B on MAT/IL-1β response in pyrogenic HSA
Each sample was incubated with whole blood with and without polymyxin B, and the IL-1β response in the supernatant was determined by ELISA. Batch A: Clean HSA sample spiked with 10 EU/ml (assay control); Batches G, H and I failed the Rabbit Pyrogen Test. Negative control: pyrogen-free physiological saline. All HSA samples were assayed at 1/4 dilution. Data are represented as mean ±SD of two separate experiments. Groups with different letters were significantly different at p<0.05

ly. Furthermore, OD values above 2 units were normally out of the linear phase of the dose response curve for both cytokines' standard curves under our assay conditions (plots not shown). IL-6 was not detected (less than 1 pg/ml), and IL-1β was 43 pg/ml. Considering samples were diluted at least 32 times to overcome interferences in the MAT/IL-6 assay and the additional sample dilution in the assay system, we could assume that differences in estimated endotoxin concentration between both readouts were due neither to the induction of IL-6 secretion by IL-1β, nor by inhibition of IL-1β production by IL-6.

Differentiation between endotoxin and non-endotoxin pyrogen contamination
In this experiment we attempted to define the contaminating source in the three batches of HSA that were pyrogenic as determined by RPT. Pool et al. (1999) demonstrated that using Polymyxin B (PMB) combined with MAT allows differentiation between endotoxin and non-endotoxin pyrogenic contamination of pharmaceuticals. PMB efficiently inhibits the cytokine response to LPS, including LPS from PMB-resistant strains of bacteria, by promoting a change in the aggregate state of LPS into an inactive LPS micelle (Gutsmann et al., 2010).

We first evaluated the effect of PMB in our assay conditions in a clean batch of HSA spiked with 10 EU/ml (Batch A in Fig. 1). There were no significant differences (p<0.05) in cytokine production between endotoxin-spiked HSA treated with PMB and the assay control; thus demonstrating that endotoxin activity was completely abrogated. By using the same approach, PMB reduced between 83 and 90% of the cytokine response in the contaminated batches (Fig. 1), indicating that they were largely contaminated with endotoxin. Nevertheless, a low cytokine re-

sponse significantly different (p<0.05) to the assay control remained in the presence of PMB. This appears to be derived from the presence of non-endotoxin pro-inflammatory entities.

4 Discussion

Testing of HSA for pyrogens has been included in several studies to benchmark the replacement of the rabbit pyrogen test with the LAL (Bleeker et al., 1985; Hegedüs and Benkö, 1977; Hochstein et al., 1979; Watt et al., 1979) and the MAT (Poole et al., 1988a,b; Taktak et al., 1991). The LAL has proven to be a very sensitive and effective assay to detect endotoxin from Gram negative bacteria. It has been employed with success for more than 20 years as an end-product endotoxin test in parenteral pharmaceuticals. However, pyrogenicity testing of HSA is still regulated mainly by the RPT, despite some drawbacks of this assay with regard to testing this product (Hartung et al., 2001). In the present study, sixteen batches of 20% HSA were assayed by LAL, RPT, and WB-MAT determining the IL-1β and IL-6 response in order to provide parallel testing data to support the safety and reliability of MAT against the established LAL and RPT.

It was found that all HSA batches were contaminated with (1,3)-β-glucans as shown by the differences found between conventional and endotoxin-specific LAL assay. The main source of contaminating (1,3)-β-glucans in blood-derived products are cellulose membranes commonly used for clarification (Buchacher et al., 2010; Nagasawa et al., 2003; Ochiai et al., 2010; Ohata et al., 2003; Usami et al., 2002).

Previous studies have indicated that the endotoxin concentration in HSA preparations determined with the ES-LAL reagent

correlates with the RPT result, and as the endotoxin spikes were recovered within the accepted range, the authors concluded that ES-LAL could be suitable for testing HSA for endotoxins (Asakawa et al., 1994; Fujiwara et al., 1990). The same has been described for other blood-derived products (Naito et al., 1992, Ochiai et al., 2010). The LAL assay is implemented in Japan for pyrogen testing of HSA (Ministery of Health and Welfare, Japanese government, 2006).

However, we encountered problems with endotoxin recovery in the interference test with both C-LAL and ES-LAL that invalidate the use of LAL as an end-product endotoxin test. Failure to recover endotoxin from HSA with LAL has been documented previously (Hochstein et al., 1979). Those authors suggested that HSA masks certain amounts of endotoxin until saturation occurs and therefore the LAL test is not able to fully detect the endotoxin spikes (Hochstein et al., 1979). The masking effect of LPS by HSA occurs by non-electrostatic interaction between both molecules, hindering the 4'-phosphate group essential for LAL reactivity inside the aggregate (Jürgens et al., 2002). This could explain why the only batches in which the spike was recovered in the interference test with ES-LAL were those that were pyrogenic in the RPT.

On the other hand, we have found an enhanced response of monocytes to LPS in the MAT/IL-6 interference test, which is probably due to the presence of cellulose-derived (1,3)-β-glucans. Similar findings have been described earlier (Kikkert et al., 2007). Conversely, others have found that glucans from *C. albicans* suppress the LPS-induced IL-6 response (Nakagawa et al., 2003). The biological activity of (1,3)-β-glucans in vertebrates is complex and relates to their origin, size, molecular structure, purity, and cell type or receptor(s) involved (for reviews see Brown, 2006; Goodridge et al., 2009; Reid et al., 2009; Tsoni and Brown, 2008).

In the current study, it also was found that endotoxin equivalent concentrations obtained with MAT/IL-6 were usually higher than with MAT/IL-1β, which could be due to (1,3)-β-glucans having directly elicited an IL-6 response in monocytes. Our thinking is based on previous reports stating that glucans of intermediate or low molecular weights could induce an IL-6 response but not an IL-1β response (Brown and Gordon, 2003, 2005). We have not found information on the molecular weight of (1,3)-β-glucans extracted from cellulose filters. However, they might be similar to those extracted from cellulose membrane components of hollow-fiber hemodialyzers, which have a molecular weight of around 24,000 (Pearson et al., 1984, 1987). This issue is also contradictory in the literature, because other reports indicate that glucans from cellulose filters (Kikkert et al., 2007) or from yeast (Nakagawa et al., 2002) do not induce IL-6 release in monocytes or monocytoid cell lines. This issue would require further studies.

Here we have found that, in a similar fashion to the conventional LAL, glucan contamination can provoke two different results in MAT/IL-6: 1) false positive for endotoxin, and 2) enhanced response to endotoxin.

The direct IL-6 response to glucan contamination by monocytes in MAT/IL-6, along with its higher sensitivity, were responsible for the saturating response in less diluted, un-spiked samples observed in MAT/IL-6 interference test (Tab. 2). As IL-6 is a well-known endogenous pyrogen, it would be conceivable that such burst release could bring about a pyrogenic episode. Yet, many of the HSA samples were contaminated with glucans and were non-pyrogenic in rabbits, probably because much higher levels of IL-6 than IL-1β are required to produce fever in humans and in rabbits (Dinarello, 2004).

It has already been stated that (1,3)-β-glucans are not pyrogenic (Roslansky and Novitsky, 1991), including those extracted from cellulose material (Buchacher et al., 2010; Pearson et al., 1984). Consequently, WB-MAT using the IL-1β readout seems to resemble the pyrogenic response in rabbits better than IL-6.

Even though clinical data demonstrating a pyrogenic effect of (1,3)-β-glucans in humans is still lacking, we should be aware that these are biologically active molecules which, like endotoxins, are classified as pathogen-associated molecular patterns (PAMP) (Medzhitov and Janeway, 2000). Therefore, as suggested by others (Kikkert et al., 2007), their presence as contaminants in pharmaceuticals could be undesirable. Further, their synergistic effect on the IL-6 response to LPS by monocytes, even at sub-threshold pyrogenic response levels of LPS, could imply a serious deleterious effect if this combination is administered to patients, which may not necessarily be revealed by a fever reaction. Currently, however, there is no explicit regulation concerning the presence of this contaminant in pharmaceuticals.

Earlier reports described the LAL assay as failing to detect pyrogenic HSA probably contaminated with non-endotoxin pyrogens as assessed by MAT (Pool et al., 1999; Taktak et al., 1991). However, the three pyrogenic batches identified by RPT in the current study were contaminated with endotoxin and one of them passed the LAL assay, whereas all three failed MAT using IL-1β or IL-6 as readout, demonstrating a good correlation between MAT and RPT for production-related pyrogenic and non-pyrogenic HSA samples.

Dehus et al. have demonstrated that the WB-MAT response to endotoxins does not always correlate with LAL assay results (Dehus et al., 2006), probably because the basic requirements for the activation of *Limulus* hemocytes and the induction of cytokines by endotoxins in human monocytes are quite different (Brandenburg et al., 2009; Gutsmann et al., 2010).

In the current study we have shown that the WB-MAT is a suitable and reliable assay for pyrogenicity testing in HSA, which overcomes essential disadvantages of the other pyrogen tests for this product. WB-MAT is not influenced by an interaction between HSA and LPS (Brandenburg et al., 2009), and as it closely resembles the human physiological response (Hartung, 2001), it should best describe the potential activity of endotoxins in HSA formulations, providing higher safety levels. Our results, together with previous studies, support the replacement of RPT with MAT as final release test for pyrogens in HSA, and probably also in other blood-derived products and therapeutic proteins.

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