Research Article

Comparison of Pyrogen Assays by Testing Products Exhibiting Low Endotoxin Recovery

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Abstract

The use of pyrogen tests to assess the risk of endotoxin in biological products has increased recently due to concerns of some regulatory authorities about products exhibiting low endotoxin recovery (LER). Manufacturers increasingly seek to reduce the use of animals unless essential to assure patient safety. The current study compares the ability of the monocyte activation test (MAT) and the bacterial endotoxin test (BET) to the rabbit pyrogen test (RPT) to detect endotoxin spikes in samples of products shown to exhibit LER. Product samples or water were spiked with endotoxin and held for three days or tested immediately in the BET, the RPT, and two variations of the MAT at the same time. Results show high sensitivity to endotoxin of both the BET and MAT, and much lower sensitivity of the RPT, indicating that much higher levels of reference standard endotoxin are required to induce pyrogenicity in the RPT than the 5 endotoxin units (EU) per kg common threshold. The results of the BET and MAT correlated well for the detection of endotoxin spike in water. We also show that LER (masking of endotoxin) found in the BET is also seen in the MAT and RPT, suggesting that the products themselves elicit a biological inactivation of spiked endotoxin over time, thereby rendering it less or non-pyrogenic. We conclude that the non-animal MAT option is a suitable replacement for the RPT to measure spiked endotoxin in biopharmaceuticals.

1 Introduction

Pyrogens comprise a heterogeneous group of fever-inducing compounds derived from microorganisms and non-microbial substances. Pyrogen testing is a health authority expectation for initial marketed authorization approval. For lot release testing of commercial biological products, the compendial *in vivo* rabbit pyrogen test (RPT) method may be waived if an equivalent *in vitro* method has been demonstrated to be applicable, e.g., the bacterial endotoxin test (BET) (USP<85>) or a validated recombinant factor C (rFC) assay (EDQM, 2021a) with an accompanying risk assessment for non-endotoxin pyrogens (US FDA, 2012; Bolden et al., 2020; Tindall et al., 2021).

Endotoxin recovery studies (ERS) are not part of compendial endotoxin method suitability testing but are defined as supplementary studies for detection of low endotoxin recovery (LER). LER is the inability to recover at least 50% endotoxin activity over time when a known amount of endotoxin is added to an undiluted product. For cases where sample treatment to mitigate endotoxin masking does not allow successful detection in the *in vitro* method, detection of spiked endotoxin is evaluated in a biological system, such as the RPT. The challenges of the application of RPT to study masking in the BET are discussed in Chapter 5 of the PDA Technical Report No. 82 (Chen et al., 2019), which proposes underlying mechanisms and contributing factors of LER, summarizes the potential clinical impact of the LER phenomenon, presents guidelines for developing LER hold-time study design, and provides strategies for the mitigation of LER.

In this study we aimed to compare the detection of endotoxin spikes in biopharmaceutical products known to be impacted by LER in the MAT, BET, and RPT. The three product samples were chosen by manufacturers based on previous studies in which LER was observed and the unspiked samples were non-pyrogenic in an RPT.

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It has been published that different sources and preparation methods of endotoxins result in widely different responses in the BET (Kikuchi et al., 2017). We selected reference standard endotoxin (RSE) since it is (1) a primary standard for bacterial endotoxin testing; (2) the predominant material used to assign potency to control standard endotoxins (CSE) for BET assays; (3) well characterized; (4) in common use; and (5) commercially available. To understand what happens to the pyrogenicity of the endotoxin spike in the sample over time, each sample was prepared and held for three days (T3) at ambient temperature or prepared and tested immediately (T0).

For the RPT, USP<151> instructions were generally followed, although we decided to test eight animals per test article in order to obtain a larger data set, since a routine test starts with three animals and then five more are tested based on results of the first three if pyrogenicity cannot be concluded. Individual and summed results were evaluated. While 5 EU/kg endotoxin challenge is considered to produce pyrogenicity measured as an increase in body temperature in the RPT, it has been reported that for individual responses higher concentrations of endotoxin spike may be required depending on the breed of rabbit (Hoffman et al., 2005; Chen et al., 2019). We challenged rabbits with 35 EU/kg in either water or biopharmaceutical product samples. Compendial RPT methods such as in the United States Pharmacopoeia *USP<151> Pyrogens* or European Pharmacopoeia *Ph. Eur. 2.6.8 Pyrogens* (EDQM, 2020a) require dilution of products such that animals are dosed in mg/kg product to match the maximum human dose. Our study was designed such that the product samples spiked with endotoxin, when diluted to each product's human mg/kg dosing concentration, contained the same final concentration of endotoxin. By these means, all three sample preparations dosed in the RPT had identical endotoxin concentrations, and the volume dosed was adjusted to the individual animal's weight to achieve 35 EU/kg. A sample of endotoxin spiked in water was prepared alongside the product samples as a control.

A kinetic-chromogenic BET assay kit was chosen because it is one of the more common methods in clinical and commercial use and offers the benefit of quantitation against a qualified lysate/standard combination.

The *in vitro* monocyte activation test (MAT) is used to detect the presence of endotoxin or non-endotoxin pyrogens through elaboration of the pro-inflammatory cytokines interleukin-1β (IL-1β) (Bleeker et al., 1994) and interleukin-6 (IL-6) (Helle et al., 1988). MAT is a suitable replacement for RPT in the EU (refer to Ph. Eur. 2.6.30 (EDQM, 2020c) and 5.1.10 (EDQM, 2020d)). Its use for LER has been described previously (Mozier, 2019). The two most commonly used monocyte sources for MAT are human peripheral blood mononuclear cells (PBMCs) (Solati et al., 2015) and human whole blood (WB). Both are available commercially, each as pools of pre-screened donors. We performed both a MAT using PBMCs from Sanquin in combination with an IL-6 readout and a MAT using WB from Merck in combination with an IL-1β readout.

The primary objectives of our study were (1) to compare the sensitivity of BET, RPT, and MAT to endotoxin; (2) to determine the influence of three LER-impacted biopharmaceutical products over time on endotoxin recovery in these assays; and (3) to determine how well the results of the three assays correlate with each other. The goal was to evaluate whether the BET and/or MAT, which is already a Method of Analysis in the Ph. Eur., is a viable alternative to the RPT for endotoxin detection in products that exhibit endotoxin masking and can thereby avoid the use of animal testing while maintaining product safety.

2 Animals, materials and methods

2.1 Study design

In this study, three different test methodologies were performed at the same time on the same set of three product samples along with a spiked water control. The study was divided between two contract research organizations (CROs), who performed the *in vivo* and *in vitro* tests, respectively. Identical aliquots of samples at optimal storage were prepared at each facility. Further, the same RSE and the same hold time study design were followed at both CROs. Subject matter experts from the sponsoring companies were present on site during the performance of the studies.

The study included a total of 8 samples, i.e., three drug products that had previously passed the RPT and were shown to have LER plus a water control were each tested three days after preparation (T3) or immediately after preparation (T0). This was achieved by a "reverse protocol approach", i.e., by preparing the T3 samples three days before preparing the T0 samples on the test date. T3 samples were gently vortexed for 15 s, sealed, and stored at 20-25ºC in a temperature-controlled chamber until use. RSE (Cat#E700 (USP) at 10,000 EU/vial) was reconstituted with 1 mL of LAL reagent water (LRW, EndoSafe®) to reach a concentration of 10,000 EU/mL. After mixing and spiking the T3 samples (see below), the vial was sealed and stored at 2-8°C. After 3 days, the material was brought to room temperature before spiking the T0 samples.

The product samples used were: Product 1, 158.3 mg/mL, formulated with histidine, trehalose, EDTA, and polysorbate 80, BET limit 28 EU/mL; Product 2, 0.0128 mg/mL, formulated with citrate, lysine monohydrate, trehalose, and polysorbate 80, BET limit 5 EU/mL, and Product 3, 5.0 mg/mL, formulated with charged, aliphatic amino acid and surfactant, BET limit 5 EU/mL. The spike levels were designed such that dilution for RPT (in mg/kg) was according to each human dose of each

Abbreviations
BET, bacterial endotoxin test; CRO, contract research organization; CSE, control standard endotoxin; ERS, endotoxin recovery study; EEU, endotoxin equivalent units; EU, endotoxin units; IL-1β, interleukin-1β; IL-6, interleukin-6; LAL, Limulus amebocyte lysate; LER, low endotoxin recovery; LRW, LAL-reagent water; MAT, monocyte activation test; PBMCs, peripheral blood mononuclear cells; RPT, rabbit pyrogen test; RSE, reference standard endotoxin; WB, whole blood

unique product while achieving matching levels of spiked endotoxin for *in vivo* and *in vitro* testing (Tab. S1-3[1]).

LAL-reagent water (LRW) was from Lonza, isotonic 0.9% NaCl solution was Solupharm Pharmaceutical Products GmbH, and 15 mL glass tubes were Pyrokontrol, ACILA Dr. Weidner GmbH for RPT. These solutions and test tubes for BET and MAT were all certified pyrogen-free.

2.2 Rabbit pyrogen test – RPT

The RPT was performed using a hybrid breed, i.e., a cross between a European domestic rabbit (Deutsches Hauskaninchen) and New Zealand Whites. A total of 64 rabbits were used with body weights ranging from 1.6 to 2.3 kg. Experimental projects were approved by the competent authority (Government of Lower Franconia, document reference RUF-55.2.2-2532-2-584-15) according to the German Animal Welfare Act. The RPT was performed using a protocol that met requirements of both *USP<151> Pyrogen Test* and *Ph. Eur*. 2.6.8 *Pyrogens* (EDQM, 2020a). The study is reported in accordance with the ARRIVE guidelines 2.0 (Percie du Sert, 2020).

RPT sample treatments were based on the known maximum human dosage of each company's product in mg/kg (Tab. S1-2[1]). All rabbits passed the sham test using pyrogen-free sterile 0.9% NaCl (sham test performed within 3 days of test initiation). The inclusion of the sham test introduces more stringent criteria for suitability of the animal to mitigate false positive results.

Doses were administered to 8 rabbits for each sample within 4 min from the start of injection. Each dose contained a spike of 35 EU/kg dose (3 mL/kg). Temperature monitoring was initiated 90 min prior to injection of test sample. Temperature sensing probe depth was not less than 7.5 cm and not more than 9 cm. Temperature monitoring data included monitoring for determination of control temperature. Temperature monitoring results at each time point were reported for all animals; the interpretation of results was not reported by the testing lab (e.g., non-pyrogenic, pyrogenic).

2.3 Bacterial endotoxin test (BET) and monocyte activation test (MAT)

The three *in vitro* tests performed were the BET using the kinetic chromogenic Limulus amebocyte lysate (LAL) test, according to current USP<85> and Ph. Eur. 2.6.14 (EDQM, 2020b), the MAT using Sanquin reagents (M2016 MAT Cell Set, pMAT cells (cryopreserved, pooled from 4 donors), and MAT culture medium supplements, M1916 Pelikine Compact Human IL-6 kit and M1980 Pelikine Tool Set from Sanquin, Amsterdam, The Netherlands), and the MAT using Merck KGaA reagents (1.44155.0001: PyroDetect Cryoblood (cryopreserved human WB from 8 donors), KHC0011: IL-1 Beta Human ELISA Kit, E0150000: PyroDetect Endotoxin Standard, Iscove's Modified Dulbecco's medium (IMDM) with L-glutamine and 25mM HEPES).

Because the goal of this study was to test all samples simultaneously as close in time as possible, out of the same sample container, the BET and the MAT were tested once, and replicates within each test (n = 2 for BET and n = 3 for MAT, as explained below) were included to reduce variation by averaging results for a given sample dilution.

BET

BET samples were diluted 10, 40 and 400-fold prior to testing to ensure lack of interference (Tab. S4[1]). The dilutions were selected based on the nominal concentration of the spiked RSE and the endotoxin standard curve of the kinetic-chromogenic LAL assay (0.005-5.0 EU/mL) and tested in duplicate.

MAT

For the MAT, multiple dilutions were tested to ensure relevant data was captured due to steep slopes with this methodology, the utilization of two MAT systems, and the sensitivity of donor cells. In this study, WB pooled from 8 donors or PBMCs pooled from 4 donors was used to test the samples. Experimental work was conducted according to the CRO's SOP described herein. The calculation and interpretation of the results was performed as detailed in Ph. Eur. 2.6.30 (EDQM, 2020c).

For the Sanquin MAT, IL-6 was measured in the supernatant after the samples were incubated overnight in thawed PBMCs (correlation coefficient of curves: 1.000 and 0.994). For the Merck MAT: IL-1β was measured in the supernatant after the samples were incubated overnight in thawed WB (correlation coefficient of curves: 0.938 and 0.947). Note that at the time of the MAT experiments both kits utilized fetal bovine serum (FBS), but non-animal alternatives are now available (Molenaar-de Backer et al., 2021). Antibodies for ELISA kits were produced recombinantly.

Samples and LRW were all tested with identical spike levels (Tab. S5[1]) to allow direct comparison of the effect of the different products (and LRW) on endotoxin activity in the MAT. The range of levels tested was based on preliminary evaluations of endotoxin in water when tested in the cell preparations for the two iterations of MAT. To test a full set of RSE standards and all sample dilutions on one plate, samples were each tested in triplicate (Tab. S6[1]). One plate included all the T0 sample dilutions and the other all the T3 sample dilutions.

3 Results

3.1 BET results

The BET results of the samples that resulted in a signal within the standard curve range were multiplied by the dilution factor and averaged as shown in Table 1. All standard curves achieved

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Tab. 1: Bacterial endotoxin testing (BET) results (values are average of n = 2)
Percentage spike recovery < 50% (marked in red) indicates low endotoxin recovery (LER).

	Dilution	EU/mL	
		T0	T3
LRW	10	8.6449	6.294
	40	13.9082	10.3684
	400	23.3872	19.8752
	Mean	**15.3**	**12.2**
Product 1	10	4.9341	2.4915
	40	6.4339	2.6266
	400	7.7345	3.2167
	Mean (% recovery)	**6.4** (42%)	**2.8** (23%)
Product 2	10	9.5892	0.0924
	40	12.4986	0.2624
	400	6.1949	< 2.0
	Mean (% recovery)	**9.4** (62%)	**0.2** (1%)
Product 3	10	2.0936	0.0537
	40	2.0447	< 0.2
	400	2.1625	< 2.0
	Mean (% recovery)	**2.1** (14%)	**0.1** (< 1%)

$R^2 \geq 0.980$. The endotoxin recovery in the spiked LRW control is expected to be within 50%-200% of the theoretical endotoxin value of 19.7 EU/mL, and results showed 78% (T0, 15.31 EU/mL) and 62% (T3, 12.18 EU/mL).

Average values for product samples were calculated as a percentage of the LRW controls (% recovery, separately for T0 and T3). In one case (Product 3, T3), only a single result was in range, so that value was used for this calculation.

The data show LER in Product 1 and Product 3 at T0 (< 50% recovery of endotoxin spiked into sample). All three products showed LER at T3 (< 1% to 23%), which was expected since these samples had previously exhibited LER. The results confirm that the products exhibit LER in the BET method.

3.2 RPT results

A notable temperature rise in the RPT is defined as an individual rise in temperature equal to or greater than 0.5°C above the animal's respective control temperature. The results in Table 2 are presented according to USP<151>, where 3 rabbits would be tested initially to determine if any individual responds with a temperature increase of 0.5°C or greater (in bold). Based on the first 3 animal responses, six of the eight samples would trigger testing in five further animals, and these six fail the pyrogen test (sum of temperature increase in 8 rabbits > 3.3°C or three rabbits with a temperature increase > 0.5°C). Notably, all three products at T0 are more pyrogenic than the LRW. Product 2 (T3) and Product 3 (T3) passed the RPT based on the first 3 or all 8 animals even though they were spiked at the same endotoxin concentration as the other samples.

Tab. 2: Rabbit pyrogen test (RPT) results: Increase in rabbit body temperature (°C) upon treatment with samples
Increased body temperature ≥ 0.5°C and sum of increases in body temperature ≥ 3.3°C are marked in bold.

	LRW T0	LRW T3	Product 1 T0	Product 1 T3	Product 2 T0	Product 2 T3	Product 3 T0	Product 3 T3
Rabbit 1	**0.5**	**0.8**	**1.0**	**0.9**	**1.0**	0.3	**1.1**	0.2
Rabbit 2	0.4	**0.6**	**0.7**	**0.6**	**1.0**	0.1	**1.0**	0.1
Rabbit 3	0.1	**0.7**	**0.7**	**1.1**	**1.1**	0.1	**0.5**	0.2
Rabbit 4	**0.5**	0.4	**0.8**	**0.9**	**0.9**	0.1	**0.6**	0.2
Rabbit 5	**0.7**	0.4	**0.8**	**0.8**	**0.7**	0.1	**1.0**	0.1
Rabbit 6	0.4	**0.5**	**0.6**	**1.0**	**0.9**	0.2	**1.1**	0.2
Rabbit 7	0.4	**0.9**	**0.7**	**0.9**	**0.9**	0.1	**0.7**	0.1
Rabbit 8	0.3	0.6	**0.8**	**0.8**	**0.5**	0.2	**1.1**	0
Sum (n = 8 rabbits)	**3.3**	**4.9**	**6.1**	**7.0**	**7.0**	1.2	**7.1**	1.1
RPT result	FAIL	FAIL	FAIL	FAIL	FAIL	PASS	FAIL	PASS

Tab. 3: Monocyte activation test (MAT) results: MAT PBMCs, IL-6 and MAT WB, IL-1β
Products were tested in both versions of the MAT. Cytokine signals are reported as endotoxin equivalent units per mL (EEU/mL) and multiplied by the dilution factor as suggested in Ph. Eur. 2.6.30 (EDQM, 2020c). Sample results below or above the standard curve range are reported as less (<) or more (>) than, respectively. Values are average of n = 3. #, results excluded from calculations as they were clearly out of trend with other dilutions and representative of a spurious assay contamination result (replicates: 0.074, 0.089 and out of range).

	Dilution	EEU/mL PBMCs, IL-6		EEU/mL WB, IL-1β	
		T0	T3	T0	T3
LRW	49	49	31.4	11.4	14.2
	66	20.5	35.8	12.1	18.9
	99	21.6	31.3	13.1	11.4
	131	23.1	39.7	15.9	18.5
	197	15.2	33.6	8.9	12.4
	263	32.1	42.1	8.5	19.2
	Mean	22	36	12	16
Product 1	49	8.33	13.9	> 98	34.3
	66	10.43	15.6	36.7	49.8
	99	13.66	17.2	42.7	47.2
	131	18.6	22.4	22.9	44.3
	197	24.41	23.6	38.8	55.8
	263	29.46	36.0	29.7	60.8
	Mean (%recovery)	18 (79%)	21 (60%)	34 (293%)	49 (309%)
Product 2	49	< 0.76	< 0.76	< 1.5	< 1.5
	66	5.68#	< 1.02	< 2.1	< 2.1
	99	< 1.54	< 1.54	< 3.1	< 3.1
	131	< 2.04	< 2.04	< 4.1	< 4.1
	197	< 3.07	< 3.07	< 6.2	< 6.2
	263	< 4.1	< 4.10	< 8.2	< 8.2
	Mean (%recovery)	< 1 (< 4%)	< 1 (< 2%)	< 2 (< 17%)	< 2 (< 13%)
Product 3	49	5.93	< 0.76	4.7	< 1.5
	66	7.79	< 1.02	3.0	< 2.1
	99	9.90	< 1.54	3.7	< 3.1
	131	12.84	< 2.04	< 4.1	< 4.1
	197	8.27	< 3.07	< 6.2	< 6.2
	263	< 4.10	12.6#	< 8.2	< 8.2
	Mean (%recovery)	9 (40%)	< 1 (< 2%)	4 (32%)	< 2 (< 13%)

Interestingly, 5 of 8 rabbits receiving the LRW T0 sample and 3 of the rabbits receiving the LRW T3 sample did not respond with a notable temperature increase. These findings suggest that 35 EU/kg is inadequate to reliably produce fever in these rabbits, even in the water control where no sample effects are present. Another observation is that none of the samples produced very high fevers in individual animals (none increased by more than 1.1°C, Tab. 2). In summary, of the 8 samples spiked with the same concentration of endotoxin, two (Products 2 and 3 both at T3) passed the USP<151> individual animal criteria in the 3-rabbit or 8-rabbit test.

3.3 MAT results

Products were diluted serially and tested in both versions of the MAT. Sample results were interpolated from an RSE standard curve performed in each assay on the same microplate (Tab. S6, Fig. S1-S4[1]). For this reason, the cytokine signals (IL-6 for PBMCs, IL-1β for WB) are reported as endotoxin equivalent units per mL (EEU/mL) and multiplied by the dilution factor as suggested in *Ph. Eur.* 2.6.30 (EDQM, 2020c). Sample results below or above the standard curve range are reported as less (<) or more (>) than, respectively.

Results corrected for dilution were averaged using values within range of the standard curve and are shown in Table 3. Two results were excluded from calculations in the PBMC, IL-6 MAT (Product 2, T0 at 66-fold dilution and Product 3, T3 at 263-fold dilution, both marked with #) as they were clearly out of trend with other dilutions and representative of a spurious assay contamination result (replicates: 0.074, 0.089 and out of range). This demonstrates the value of multiple dilutions, where anomalies can be identified in a series.

In the PBMC, IL-6 MAT, the spiked LRW samples versus nominal spike concentration (19.7 EU/mL) were 22 EEU/mL (T0) and 36 EEU/mL (T3). This variation between plates is approximately 2-fold and not unusual for these types of *in vitro* assays. To account for this variation within the experiment, sample results were calculated as percent of the spiked LRW sample on the same plate. Product 1 showed 79% recovery at T0 and 60% at T3; Product 2 and Product 3 both showed lower recovery at T0 (< 4% and 40%, respectively) and at T3 (both samples were < 2%).

In the WB, IL-1β MAT, the spiked LRW sample produced results of 12 and 16 EEU/mL, within the expected nominal spike concentration of 19.7 EU/mL, as shown in Table 3. Product 1 is shown to have high recovery at T0 (293%) and no change after 3 days in the T3 sample (309%). Product 2 had undetectable signal and no measurable recovery either at T0 or T3. Product 3 had low recovery (32%) at T0 and no measurable recovery at T3 (< 13%).

3.4 Correlations among the assays

The results of all the assays are summarized in Table 4. The LRW controls tested in the MAT WB assay produced results (12-16 EEU/mL) that agreed with nominal endotoxin spike level (19.7 EU/mL) and the measured BET levels (12-15 EU/mL). LRW controls in the MAT PBMC assay gave similar concordance, although it is interesting that the higher T3 (36 EEU/mL) is the same condition that showed more pyrogenicity in the RPT. Overall, the results in LRW confirm (1) the suitability of MAT as a correlate to BET for the highly purified RSE; and (2) the relative insensitivity of these rabbits to RSE.

Product 1, when tested immediately after spiking (T0), is already less than half as active as the spiked control in the BET, suggesting a masking effect on RSE that is rapid; however, at T3 it loses only about half again of the activity. It is more pyrogenic than the spiked control at T0 in the RPT, and the pyrogenicity is maintained until T3. Essentially, the activity in the BET and RPT does not change significantly over the three days in this sample, suggesting that whatever changes are caused by the sample to the spiked RSE are immediate. Interestingly, in the MAT WB, IL-1β assay the recovery versus control of spiked RSE is 2-3-fold higher in Product 1 at T0 and increases by T3, but is unchanged in MAT PBMC, IL-6. Further studies have revealed that unspiked Product 1 can elicit a MAT signal (data not shown), suggesting that a non-endotoxin substance may be present and signaling in the MAT.

Tab. 4: Summary of data from BET, RPT, and MAT assays
Values indicating < 50% recovery (LER) or passing RPT (n = 8, USP<151>) are marked in red.

	BET [EU/mL]	**RPT** (sum n = 8) [°C]	**MAT PBMC, IL-6** [EEU/mL]	**MAT WB, IL1β** [EEU/mL]
LRW (T0)	15.3	3.3	22	12
LRW (T3)	12.2	4.9	36	16
Product 1 (T0)	6.4	6.1	18	34
Product 1 (T3)	2.8	7.0	21	49
Product 2 (T0)	9.4	7.0	< 1	< 2
Product 2 (T3)	0.2	1.2	< 1	< 2
Product 3 (T0)	2.1	7.1	9	4
Product 3 (T3)	0.1	1.1	< 1	< 2

Product 2 is active in BET at T0 but is no longer active at T3, suggesting a time-dependent masking process. The same is seen in the RPT. Product 2 induces neither IL-6 nor IL-1β in the MAT at either timepoint, indicating a masking effect in human cells.

Product 3 shows immediate masking at T0 in the BET. However, it is still active in the RPT at T0 but is masked by T3 (non-pyrogenic per USP<151> criteria). It induces little IL-6 or IL-1β release in the MAT at T0 and is completely masked at T3. Overall, Product 3 exhibits consistent masking in all tests at T3.

Note that for the T3 values, the MAT and RPT results correlated for all products, indicating that both are adequate for assessing the activity of masked endotoxin. BET and RPT correlate for Product 2 and 3 but not for Product 1. Because this sample was shown to signal in MAT in the absence of RSE, it is speculated that the non-endotoxin pyrogen may act in synergy with masked endotoxin.

4 Discussion

We have confirmed that, in these rabbits, significantly more than 5 EU/kg is needed to reliably induce pyrogenicity as

even 35 EU/kg did not induce a pyrogenic response in all rabbits. This phenomenon has been observed by others (Chen et al., 2019) and contrasts with larger studies where 5 EU/kg was able to reliably produce pyrogenicity (Hoffman et al., 2005). Criteria for types of rabbits (breed, strain) are not defined for the RPT, nor is a minimum sensitivity to endotoxin required by the compendia. Although the higher pyrogenicity in RPT at T3 in the LRW samples correlates with increased MAT signals in both WB and PBMCs at T3, a mechanism is not known (nor hypothesized in literature). These data suggest that the MAT is a suitable alternative to the RPT for purified endotoxins in water.

Regarding sensitivity of all assays to endotoxin, the RPT barely detects 35 EU/kg (11.7 EU/mL) in LRW, whereas MAT is at least 2 orders of magnitude more sensitive (at least 0.1 EU/mL), and BET is the most sensitive (at least 0.01 EU/mL). The MAT is more relevant for drug evaluations for human use over both BET and RPT as it is based on a human tissue response. Our study only evaluated one highly purified endotoxin, but it has been shown by others that the MAT detects rabbit pyrogenicity from a broader diversity of endotoxins than the BET (Fennrich et al., 1999).

The findings in spiked samples show that there are differences between products with LER, indicating that either the active ingredient or the formulation components change the structure or availability of spiked endotoxin. As expected, the recovery of spiked endotoxin was lower in all samples (than in LRW) and decreased further over the 3-day hold. Clearly, the T0 samples are less relevant to biomanufacturing, since a real contamination would likely occur at a step in the process well ahead of final sample collection and storage. It should also be noted that the spiked endotoxin is purified, of just one type of the myriad endotoxins that can occur in nature, and thus not likely fully representative of more naturally occurring forms as described by others (Chen et al., 2019).

Masking in the BET occurs immediately upon spiking (T0) in all samples and continues over the hold time, likely a perturbation of the physico-chemical state of the spiked RSE. It is clear that the inactivation of endotoxin activity in the BET (the conventional *in vitro* test employing interaction with Limulus amoebocyte lysate) also occurs in the MAT and the RPT assays for two of the products (2 and 3). These data suggest that undetected endotoxin, if present in Products 2 and 3 and not fully detected by BET, would be inactivated and not biologically active in humans (both MAT tests were negative at T0 and T3). However, Product 1, which was negative in the BET, was pyrogenic in RPT and positive in MAT, suggesting it may benefit from orthogonal testing beyond BET (e.g., Kikkert et al., 2007). In all cases where pyrogenicity was detected in the RPT, it was also detected in the MAT. These observations suggest that MAT is a viable alternative to RPT for confirmation of the absence of pyrogenicity, especially in cases where endotoxin demasking is not successful using the tools recommended in PDA TR82 (Chen et al., 2019).

Fortunately, most products do not have this LER issue, but when they do, based on our results, the MAT is a technology that is more sensitive and suitable as a pyrogenicity test. Finally, although more development effort and expertise is required for the MAT, it is shown to be suitable as a replacement for the RPT. Based on human tissue, the MAT has the advantage of representing the human response, an improvement over the rabbit test. Thus, the MAT is an appropriate analytical tool to support the European Directorate for the Quality of Medicines and HealthCare (EDQM) 5-year goal to eliminate the RPT completely from the Ph. Eur. (EDQM, 2021b).

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Conflict of interest

Authors confirm they have no potential conflicts of interest.

Data availability

The original data of this study is retained by Pfizer, Amgen and Roche and available only by permission.