EUROPEAN COMMISSION
DIRECTORATE GENERAL JRC
JOINT RESEARCH CENTRE
Institute for Health and Consumer Protection JRC
European Centre for the Validation of Alternative Methods (ECVAM)

Ispra, 24 Mar. 06

Response to the ICCVAM-PWG Questions for ECVAM regarding the five in vitro pyrogenicity test method submissions

Background

The concept of pyrogen testing employing the human fever reaction has been first proposed 25 years ago by Dinarello et al., when the mediators of the fever reaction were identified and could be used as a measure of the response to pyrogens. Since, a variety of test systems employing this reaction has been proposed and used in more than 100 institutions. Over the last decade, about 5 million $/€ of public funding by the EU, the German BMBF, ZEBET and ECVAM enabled the standardization and formal validation of the most eminent approaches.
Following an ECVAM workshop in 2000 (Hartung et al. 2001), a validation study was organized, which targeted solely the replacement of the rabbit animal test. The lower limit of detection of these assays compared to the rabbit and the fact that the novel assays can be used in a quantitative manner was not addressed in this validation study. The study also addressed specifically products, which are tested in rabbits, i.e. intravenous parenterals with a focus on those not testable in the Limulus amoebocyte lysate (LAL) assay/bacterial endotoxin test (BET). Since both the rabbit test and LAL/BET are mainly reactive to Gram-negative endotoxin, the study focused on this predominant endotoxin. However, outside the validation study a wealth of scientific information, especially on the human whole blood pyrogen tests, is available, which was referred to in the dossiers.
The developing laboratories had been held responsible for the content of the individual dossiers, which were commissioned by ECVAM. They were contacted to provide information to respond to the questions of the ICCVAM-PWG. The respective responses are compiled below. Editorial changes as suggested will be carried out on a short term.

1. In the BRDs, the accuracy evaluation is based on the application of a prediction model derived from historical in vivo rabbit data. However, if the proposed test methods are intended as replacements for the current pyrogenicity test methods (i.e., rabbit pyrogen test; BET), should not the accuracy analysis be a direct comparison of the proposed in vitro methods with both of these reference test methods? Can you provide data from such a comparison for review?

Due to ethical and legal reasons, it was not possible to perform the rabbit pyrogen test in parallel to the in vitro methods. The development of the prediction model was therefore based on historical rabbit data. Since rabbit strains differ in their sensitivity, data generated in the most sensitive rabbit strain were used. 60 years of pharmcopoeial use of the rabbit assay has shown, that the limit concentration is effectively protecting humans. The availability of a WHO reference material also allowed basing the study on a historic comparison.

On the basis of the determined rabbit fever threshold (Hoffmann S, Luderitz-Puchel U, Montag-Lessing U and Hartung T. Optimisation of pyrogen testing in parenterals according to different pharmacopoeias by probabilistic modelling, J. Endotoxin Res. 2005, 11:25-31), it was possible to model the performance of these rabbits, when testing the samples of the validation study assuming no additional interference of the samples. The sensitivity of the rabbit pyrogen test is 57.9% and the theoretical specificity of the rabbit pyrogen test is 88.3%. Unfortunately these numbers have not been correctly reported in the BRD, in sections 4.2 and 4.6., where the last sentence reads " .. sensitivity of the rabbit pyrogen test is 75.04% and the theoretical specificity of the rabbit pyrogen test is 95.80%.

Joint Research Centre · I-21020 Ispra (VA), Italy · TP 202
Telephone: direct line (+39-0332) 785939, · Telefax: 786297
E-mail Internet:thomas.hartung@cec.eu.int
WWW: http://www.jrc.cec.eu.int/

Comparisons between rabbit and human tests were also carried out by establishing a rabbit whole blood assay (Schindler S et al. Comparison of the reactivity of human and rabbit blood towards pyrogenic stimuli. ALTEX 2002, 20:59-63). The study reflected the slightly lower sensitivity of rabbit towards endotoxins and discrepancies for non-endotoxin pyrogens.

Based on historic rabbit results, it has been shown that the enormous differences in potency of endotoxins (4 log orders) from different bacterial species are reflected in both the rabbit and the WB/IL-1 but not in the BET (Fennrich S et al. Detection of endotoxins and other pyrogens using human whole blood. Dev. Biol. Standards, 1999, 101:131-139). The BET can thus not be used as a quantitative measure of endotoxicity to mammals if the bacterial species is not known. This has been recently expanded (Dehus O, Hartung T, and Hermann C. Pseudomonas endotoxin, a prominent contamination of water samples, is strongly overestimated in the Limulus Amebocyte Lysate Assay with regard to its pyrogenicity. J Endotox Res 2006, in press.) to endotoxins of Pseudomonas, which are overestimated by the BET by 2-3 log orders.

Although, the BET was not addressed in the validation study, it has several well-known limitations, which might in the future lead to additional uses of the novel tests:

- BET is restricted to Gram-negative endotoxin and misses those from Gram-positive bacteria or fungi
- BET it is disturbed by many components such as endotoxin-binding proteins (ample e.g. in blood-derived medicines), lipophilic substances, glucans present in herbal medicinal products or derived from cellulose filters; in fact, about 90% of LAL testing is done on water samples only due to these limitations
- BET does not reflect the biological potency of different endotoxins in humans (discrepancies up to 10.000fold)
- BET consumes animals (several recent studies indicate 15% mortality of bled animals to be seen in the light of about 60% reduction in horseshoe crab landings between 1998 and 2003)
- BET does not work for solid materials (medical devices) or cellular therapies without difficult extraction procedures
- BET does not work for air-born pyrogens increasingly recognized as a health threat, because of the restriction to Gram-negative endotoxins, not reflected endotoxin potency and impossibility to measure directly on air filters

Within the validation study, two samples of a human serum albumin (HSA) were provided to the developing labs. One of these was essentially pyrogen-free, the other one was a contaminated lot that was associated with adverse reactions in recipients. The labs themselves were responsible for the testing and supply of the results. Two labs, responsible for the THP-1/TNF and the PBMC/IL-6 methods, did not report results for this phase. Generally, the clean lot was used to determine an interference free dilution of the HSA. Then, although dilution response curves were also produced for the contaminated lot, the response of this dilution was assessed by means of an LPS standard curve. The same approach was taken with the two lots of Gentamicin that were provided. Again, one of these was clean and one was associated with adverse reactions in recipients. The results for the HSA and Gentamicin with regard to the discrimination of the pyrogen-free and contaminated lots are presented in the tables.

HSA	THP-1-Neo	MM6-IL6	blood-IL6	blood-IL1	PBMC-IL6
Interference free dilution	1:4	1:2	1:4 – 1:8	1:4	1:10
Discrimination of lots	+	+	+	-	+

Table: Results of HSA-testing for four methods

For the HSA the THP-1-Neo, MM6-IL6, blood-IL6 and PBMC-IL6 were able to distinguish the contaminated lot from the pyrogen-free lot.

Gentamicin	THP-1-Neo	MM6-IL6	blood-IL6	blood-IL1	PBMC-IL6
Interference free dilution	not testable	1:4	1:1 (2 donors)*	1:10	1:16
Discrimination of lots	-	+	+	+	+

Table: Results of Gentamicin-testing for four methods (* one donor not suitable)
For Gentamicin only the THP-1-Neo could not distinguish the lots because of severe interference. Although the interference free dilutions differed, the other four methods could discriminate the pyrogen-free and the contaminated lot.

Outside the validation study, direct comparisons have been reported for the blood-IL-1 test (referred to as IVPT) by the German National Control Authority, the Paul-Ehrlich-Institute (PEI), to the European Pharmacopoeia:

In vitro Pyrogen Test (IVPT) in comparison to Rabbit Pyrogen Test (RPT) and Limulus Test (LAL)

a) Fever reactions caused by a batch of Human Serum Albumin
(negative in RPT as well as in LAL test)

After application of a defined batch of Human Serum Albumin, fever reactions have been reported to the PEI. The batch has been withdrawn from the market. The manufacturer had tested the product in RPT as a release criterion with negative result.

The PEI examined samples of the batch in RPT, in IVPT (Whole Blood Pyrogen Test), and in LAL. Negative Albumin batches of the same manufacturer served as controls.

RPT as well as LAL remained negative. The results of Whole Blood Pyrogen Test (5 different donors, at least 15 repetitions per donor) are shown in table2.

Table: Incriminated Human Serum Albumin

Donor	Incriminated batch IL-1 (pg/ml)	Control batch IL-1 (pg/ml)	Quotient incriminated/control
1	79.0	4.0	19.75
2	14.1	3.9	3.61
3	44.3	15.0	2.95
4	20.9	14.9	1.4
5	71.9	3.9	18.44
Mean	46.04	8.34	5.52

Remarks
A) In routine IVPT of PEI, pools from blood of 5 donors are used equalising the differences in donor reactions. The incriminated batch, negative in LAL as well as in RPT, had been clearly detected in IVPT.
B) There is an individual susceptibility towards Non-endotoxin pyrogens in humans in contrast to endotoxin that leads to fever reactions in almost all recipients.

b) Coagulation Factor VIII Concentrates
(negative in RPT and positive in IVPT)

In an early pilot study, five different Factor VIII Concentrates (one batch each) have been tested in RPT and in IVPT (Whole Blood Pyrogen Test) in parallel. The results are shown in table 2.

Table: Pilot study Factor VIII

Product	Whole Blood Pyrogen Test			Rabbit Pyrogen Test	
	IL-1 (pg/ml)	cut off (pg/ml)	result	temperature sum of 3 rabbits	result
A	130.4	18.6	positive	0.71 °C	negative
B	159.3	27.8	positive	0.70 °C	negative
C	32.6	32.6	negative	0.45 °C	negative
D	33.5	28.8	positive	1.6 °C*	negative
E	129.7	15.5	positive	0.37 °C	negative

* 6 rabbits (repetition)

It has to be taken into account that in case of F VIII – because of the small injection volume of 50 IU F VIII per kg corresponding to 500 µl per kg – the Rabbit Pyrogen Test is less sensitive (20 IU LPS/ml) than the Whole Blood Pyrogen Test (detection limit 0.25 to 0.5 IU LPS/ml).

It should be pointed out that there was a discussion in group 6B concerning the increase of injection volume of F VIII preparations in RPT even for protection of bleeding patients requiring greater amounts of F VIII.

c) Pyrogenic batch of a plasma derivative
(positive in RPT, negative in LAL test)

One batch of a partially purified, immunoglobulin containing plasma derivative was tested positive in RPT and negative in LAL by PEI. By the time the IVPT had been implemented, there was no further sample of the product available.

d) Factor VIII Concentrates, comparison study with spiked products.

Factor VIII concentrates from 6 different manufacturers (Immunate STIMplus 1000, Haemoctin SDH 1000, Octonate 1000, Fandhi 1000 IE, Beriate P 1000, and Haemate HS 1000) were tested (3 lots each) in a comparative study between RPT and IVPT (according to EP). The 18 preparations were spiked with two different concentrations of endotoxin (WHO Standard) and, thereafter, tested on the same day in parallel. Un-spiked preparations served as controls. All together, 162 rabbits were involved into the study.

The results are shown in table 3. Applying 5 IU endotoxin per kg body weight of the rabbits (representing the fever threshold of most sensitive rabbit strains) almost all rabbits responded with a temperature increase in the range allowing a repetition of the test. No positive result could be seen whereas the IVPT produced positive results in every experiment. After application of the 3-fold dose LPS in Factor VIII, the RPT identified 11 of 18 preparations clearly positive, in 7 cases the repetition range was achieved. Again, all samples in IVPT were positive. A false negative result was observed in one experiment of IVPT (1 of 4 donors); the repetition of the experiment remained negative.

Table: Comparison study Factor VIII (18 batches, 162 rabbits)

Endtotoxin (WHO Standard)		Rabbit Pyrogen Test 3 animals per test			Whole Blood Pyrogen Test n = 4 donors each	
Rabbit	IVPT	positive	repetition possible	negative	positive	negative
IU LPS / kg (1 ml / kg)	IU LPS / ml	> 2.65 °C	> 1,15 < 2.65 °C	< 1.15 °C		
0	0	0	0	18	0 (1)*	72 (71)*
5	5	0	17	1	72	0
15	15	11	7	0	72	0

* testing the same sample, the blood of 3 donors remained negative, the blood of 1 donor reacted slightly positive in the first experiment, the repetition was negative

e) Human Serum Albumins, comparison study with spiked products

Human Serum Albumins of various protein concentrations from five different manufacturers (5 % Immuno, 20 % Immuno, 20 % Kabi, 5 % Biotest, 20 % Biotest, 25 % Biotest, 5 % Centeon, 20 % Centeon, 5 % DRK BaWue, 20 % DRK BaWue) were tested (3 batches each, in case of 25 % Biotest only 2 batches were available) in a comparative study between RPT and IVPT (according to EP). The 29 preparations were spiked with two different concentrations of endotoxin (WHO Standard) and, thereafter, tested on the same day in parallel. Un-spiked preparations served as controls. All together, 261 rabbits were involved into the study.

The results are shown in table 4 (see page 4). Only 5 batches containing the borderline endotoxin concentration could be defined as positive by the RPT whereas the IVPT identified all samples. Applying the double dose, in 21 of 29 cases a clear positive result could be observed in the RPT, in 8 cases the repetition range was achieved.

Table: Comparison study Human Serum Albumin (29 batches, 261 rabbits)

Endtotoxin (WHO Standard)		Rabbit Pyrogen Test 3 animals per test			Whole Blood Pyrogen Test	
Rabbit	IVPT	positive	repetition possible	negative	positive	negative
IU LPS / kg	IU LPS / ml	> 2.65 °C	> 1,15 < 2.65 °C	< 1.15 °C		
0	0	0	0	29	0	29
5	5	5	23	1	29	0
10	10	21	8	0	29	0

It has to be pointed out that in most of the RPTs the maximal permitted injection volume of 10 ml per kg body weight has been used, which represents “worst case sensitivity conditions” of RPT (except for HSAs of 20 % and 25 % protein content where the EP allows lower volumes only). Thus, the sensitivity limit of RPT has been met in this study.

f) Fever reactions caused by an infusion solution

An infusion solution containing gelatine (release criterion LAL) induced adverse fever reactions in hospitals. The manufacturer withdrew the incriminated batches from the market and reinvestigated

them for endotoxin and, additionally, for pyrogenicity in RPT. The company observed LAL negative but RPT positive results in one batch. However, the most interesting batch which caused fever in patients remained negative in LAL as well as in rabbits. They were blinded by the manufacturer, sent to PEI, and analysed in Whole Blood Pyrogen Test. Furthermore, the PEI asked for blinded non-incriminated control batches.

Table 5: Incriminated infusion solution containing gelatine

batch	LAL test	rabbit test	fever in patients	Whole Blood Pyrogen Test		
				IL-1 (pg/ml)	IL-6 (pg/ml)	TNF □ (pg/ml)
A	negative	negative	no	8.5	28.0	28.2
B	negative	positive	yes	142.6	654.4	67.6
C	negative	negative	yes	421.5	9444.0	116.7
cut off:				32.6	127.6	43.6

The results are summarised in table 5. The incriminated batches could be identified very clearly in IVPT even the sample remaining negative in RPT. The fever causing substances have to be seen as non-endotoxin pyrogens not detectable in LAL. At least one of them is not pyrogenic for rabbits but for humans. The IVPT is basing on a “homogeneous indicator system”, the activation of human monocytes as central mediators of fever reaction. In conclusion, the IVPT indicates in vivo reactions of humans whereas the rabbit is not able to image every non-endotoxin pyrogen.

The results are in concordance with adverse fever reactions by an antibiotic (gentamycin) happened on the US market some years ago which had negative LAL test as release criterion also. The drug produced unclear results in RPT but could be tested as positive in IVPT (personal communication from Stephen Poole, NIBSC).

Remark: Unfortunately, the manufacturer of the infusion solution does not agree in publication of the results.

2. The proposed applicability domain of the test methods covers both endotoxin and non-endotoxin pyrogens. How do the validation studies included in the BRDs support both indications when there is no data provided to indicate their utility for non-endotoxin pyrogens? Can you provide data for any of the proposed test methods tested with non-endotoxin pyrogens?

Within the validation study, eleven substances provided to the developing labs of the new methods were tested. For each substance the smallest concentration (in ng/ml) inducing a response in the respective method was reported. In the table a summary of the results is presented.

	WBT/IL1	WB/IL-6	PBMC/IL-6	MM6/IL-6	THP-1/Neo
Curdlan	1000	1000	100	1000	1000
Glucan-Barley	Negative	negative	negative	negative	negative
Glucan-Yeast	not done	negative	negative	negative	negative
Zymosan	Negative	10000	10000	negative	10000
PHA-L	100	10000	100	100	1000

PHA-E	Negative	negative	negative	10000	negative
Lipid A	10000	1000	10000	1000	negative
Glucan STD	Negative	negative	negative	negative	undiluted
Endotoxin-C	4	40	0.4	4	4
Endotoxin-G	0.4	40	4	4	4
LTA	5000	500	5000	6250	1000

Table: Smallest concentration [ng/ml] or dilution of substances active in the respective method

The three glucans, which give false positive results in the BET, were not active at the highest concentration of 10000 ng/ml assessed in the five new methods in which they were tested. The Curdlan, which reacted in the BET, induced a cytokine response in the new methods generally only at a concentration of some 1000 ng/ml. Also, Zymosan and monophosphoryl-Lipid A were inactive or active only in very large doses in the new methods. The results for the PHA-L differed slightly between the methods. It was most reactive in the blood-IL1, the PBMC-IL6 and the MM6-IL6. However, the PHA-L was more reactive than the PHA-E, which was largely inactive. The potencies of the Pseudomonas endotoxins C and G differed somewhat in the different methods. The LTA was most active in the blood-IL6 and the THP-1-Neo methods. Taken together, the assays did not react to the LAL false-positive glucans and curdlan, but reacted to the LAL-false negative LTA.

WB/IL-1

The developing laboratory of the WBT tests has in fact been using this assay to identify and purify the Gram-positive counterpart, i.e. lipoteichoic acid (LTA), to Gram-negative lipopolysaccharide (LPS) in a series of studies:

1. Figuero-Perez I, Stadelmaier A, Morath S, Hartung T and Schmidt RR. Synthesis of structural variants of Staphylococcus aureus lipoteichoic acid (LTA). Tetrahedron 2005, 16:493-506.
2. Grandel U, Hopf M, Buerke M, Hattar K, Heep M, Fink L, Bohle RM, Morath S, Hartung T, Pullamsetti S, Schermuly RT, Seeger W, Grimminger F and Sibelius U. Mechanisms of cardiac depression caused by lipoteichoic acids from Staphylococcus aureus in isolated rat hearts. Circulation 2005, 112:691-698.
3. Grangette C, Nutten S, Palumbo E, Morath S, Hermann C, Dewulf J, Pot B, Hartung T, Hols P and Mercenier A. Enhanced anti-inflammatory capacity of a Lactobacillus plantarum mutant synthesizing modifed teichoic acids. Proc. Natl. Acad. Sci. U S A 2005, 102:10321-10326.
4. Henneke P, Morath S, Uematsu S, Weichert S, Pfitzenmaier M,Takeuchi O, Mueller A, Poyart C, Akira S, Berner R, Teti G, Geyer A, Hartung T, Trieu-Cuot P, Kasper DL and Golenbrock DT. Role of lipoteichoic acid in the phagocyte response to group B Streptococcus. J. Immunol. 2005, 174:6449-6455.
5. Hermann C, von Aulock S, Dehus O, Keller M, Okigami H, Gantner F, Wendel A, and Hartung T. Endogenous cortisol determines the circadian rhythm of LPS- but not LTA-inducible cytokine release. Eur J Immunol 2005, in press.

6. Hoebe K, Georgel P, Rutschmann S, Du X, Mudd S, Crozat K, Sovath S, Shamel L, Hartung T, Zahringer U and Beutler B. CD36 is a sensor of diacylglycerols. Nature 2005, 433:523-527.

7. Kinsner A, Pilotto V, Deininger S, Brown GC, Coecke S, Hartung T and Bal-Price A. Inflammatory neurodegeneration induced by lipoteichoic acid from Staphylococcus aureus is mediated by glia activation: the role of nitrosative and oxidative stress, mitochondrial dysfunction and caspase activation. J Neurochem 2005, 95:1132-1143.

8. Morath S, von Aulock A and Hartung T. Structure/ function relationships of lipoteichoic acids. J. Endotoxin Res. 2005, in press.

9. Dahle MK, Overland G, Myhre AE, Stuestol JF, Hartung T, Krohn CD, Mathiesen O, Wang JE and Aasen AO. The phosphatidylinositol 3-kinase/protein kinase B signaling pathway is activated by lipoteichoic acid and plays a role in Kupffer cell production of interleukin-6 (IL-6) and IL-10. Infect Immun 2004, 72:5704-11.

10. Lotz S, Aga E, Wilde I, van Zandbergen G, Hartung T, Solbach W and Laskay T. Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2. J. Leukoc. Biol. 2004, 75:467-477.

11. Lynch NJ, Roscher S, Hartung T, Morath S, Matsusshita M, Maennel DN, Kuraya M, Fujita T and Schwaeble WJ. L-ficolin specifically binds to lipoteichoic acid, a cell wall constituent of Gram-positive bacteria, and activates the lectin pathway of complement. J. Immunol. 2004, 172:1198-1202. (IF 6.7)

12. Mattsson E, Hartung T, Morath S and Egesten A. Highly purified lipoteichoic acid from Staphylococcus aureus induces procoagulant activity and tissue factor expression in human monocytes but is a weak inducer in whole blood-comparison with peptidoglycan. Infect. Immun. 2004, 72:4322-4326.

13. Triantafilou M, Morath S, Mackie A, Hartung T and Triantafilou K. Lateral diffusion of Toll-like receptors reveals that they are transiently confined within lipid rafts on the plasma membrane. J. Cell Sci. 2004, 117:4007-4014.

14. Triantafilou M, Manukyan M, Mackie A, Morath S, Hartung T, Heine H and Triantafilou K. Lipoteichoic acid and Toll-like receptor 2 internalization and targeting to the Golgi is lipid raft dependent. J. Biol. Chem. 2004, 279:40882-40889.

15. Zeidler D, Zahringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P and Durner J. Innate immunity in Arabidopsis thaliana: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc. Natl. Acad. Sci. U S A 2004, 101:15811-6.

16. Deininger S, Stadelmaier A, von Aulock S, Morath S, Schmidt RR, Hartung T. Definition of structural prerequisites for lipoteichoic acid inducible cytokine induction by synthetic derivatives. J. Immunol. 2003, 170:4134-4138.

17. Overland G, Morath S, Yndestad A, Hartung T, Thiemermann C, Foster SJ, Smedsrod B, Mathiesen O, Aukrust P, Aasen AO and Wang JE. Lipoteichoic acid is a potent inducer of cytokine production in rat and human Kupffer cells in vitro. Surg. Infect. 2003, 4:181-191.

18. Schröder NWJ, Morath S, Alexander C, Hamann L, Hartung T, Zähringer U, Göbel UB, Weber JR, Schumann RR. Lipoteichoic acid (LTA) of S. pneumoniae and S. aureus activates immune cells via toll-like receptor (TLR)-2, LPS binding protein (LBP) and CD14 while TLR-4 and MD-2 are not involved. J. Biol. Chem. 2003, 278:15587-94.

19. Schröder NWJ, Hermann C, Hamann L, Göbel UB, Hartung T, Schumann RR. High frequency of polymorphism Arg753Gln of the Toll-like receptor-2 (TLR-2) gene detected by a novel allele specific PCR. J. Mol. Med. 2003, 81:368-72.

20. Stadelmaier A, Morath S, Hartung T, Schmidt RR. Synthesis of the first fully active

lipoteichoic acid. Angew. Chemie Int. Ed. 2003, 42:916-920. auch: Stadelmaier A, Morath S, Hartung T, Schmidt RR. Synthese der ersten aktiven Lipoteichonsäure. Angew. Chemie 2003, 115:945-949.

21. Dalpke AH, Frey M, Morath S, Hartung T, Heeg K. Interaction of lipoteichoic acid and CpG-DNA during activation of innate immune cells. Immunobiol. 2002, 206:392-407.
22. Hermann C, Spreitzer I, Schröder NWJ, Morath S, Lehner MD, Fischer W, Schütt C, Schumann RR and Hartung T, Cytokine induction by purified lipoteichoic acids from various bacterial species – role of LBP, sCD14, CD14 and failure to induce interleukin-12 and subsequent interferon-γ release, Eur. J. Immunol., 2002, 32:541-551.
23. Jacinto R, Hartung T, McCall C and Li L. Lipopolysaccharide and lipoteichoic acid-induced tolerance and cross-tolerance: distinct alterations in IL-1 receptor-associated kinase. J. Immunol. 2002, 168:6136-6146.
24. Morath S, Geyer A, Spreitzer I, Hermann C and Hartung T. Structural decomposition and heterogeneity of commercial lipoteichoic acid preparation. Infect. Immun. 2002, 70:938-944.
25. Morath S, Stadelmaier A, Geyer A, Schmidt RR and Hartung T. Synthetic lipoteichoic acid from Staphylococcus aureus is a potent stimulus of cytokine release. J. Exp. Med., 2002, 195:1635-1640.
26. Yipp BG, Andonegui G, Howlett CJ, Robbins SM, Hartung T Ho M and Kubes P. Profound differences in leukocyte-endothelial cell responses to lipopolysaccharide versus lipoteichoic acid. J. Immunol. 2002, 168:4650-4658.
27. Lehner MD, Morath S, Michelsen KS, Schumann RR and Hartung T. Induction of cross-tolerance by LPS and highly purified lipoteichoic acid via different Toll like receptors independent of paracrine mediators. J. Immunol. 2001, 165:5161-5167.
28. Morath S, Geyer A, Hartung T. Structure/function-relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus. J. Exp. Med., 2001, 193:393-397.
29. Michelsen KS, Aicher A, Mohaupt M, Hartung T, Dimmeler S, Kirschning CJ and Schumann RR. The role of Toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells -PGN and LTA are inducers of DC maturation and require TLR2. J. Biol. Chem. 2001, 276: 22041-22047.
30. Opitz B, Schröder NWJ, Spreitzer I, Michelsen KS, Kirschning CJ, Hallatschek W, Zähringer U, Hartung T, Göbel UB and Schumann RR. Toll-like receptor (TLR)-2 mediates treponema glycolipid and lipoteichoic acid (LTA)-induced NF-kappa B translocation. J. Biol. Chem. 2001, 276:22041-22047.
31. van de Wetering JK, van Eijk M, van Golde LMG, Hartung T, van Strijp JAG and Batenburg JJ, Characteristics of surfactant protein A and D binding to lipoteichoic acid and peptidoglycan, two major cell wall components of Gram-positive bacteria. J. Inf. Dis. 2001, 184:1143-1151.

LTA and LPS were compared in the WB/IL-1:

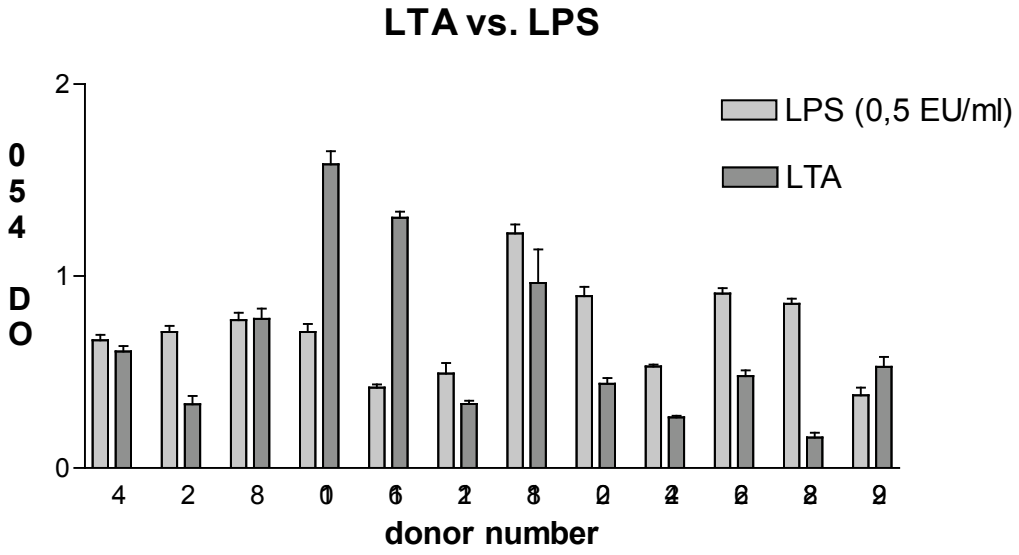


Fig. Reactivity of 12 donors towards LPS E. coli O113: H10 (0.5 EU/ml) and LTA from B. subtilis
The reactivity of 12 donors towards a challenge of 0.5 EU/ml LPS and the LTA was compared. In all cases, the stimuli tested clearly positive, although the donors did not necessarily react homogenously.

The studies were more recently expanded to fungi (Kindinger at al., A new method to measure air-borne pyrogens based on human whole blood cytokine response. J. Immunol. Meth. 2005, 298:143-153).
Fungi pose a particular health problem, since they or their spores are potentially neurotoxic, hepatotoxic, nephrotoxic and even teratogenic. The identification of pathogenic fungi in parenterals and, even more importantly, air, is therefore a particular challenge. Fungi in general proved to be highly active in the whole blood test, though differences between different strains exist (see figure). That the pyrogenic potency is not due to LPS is demonstrated in the subsequent figures employing the LPS inhibitor Polymyxin B.

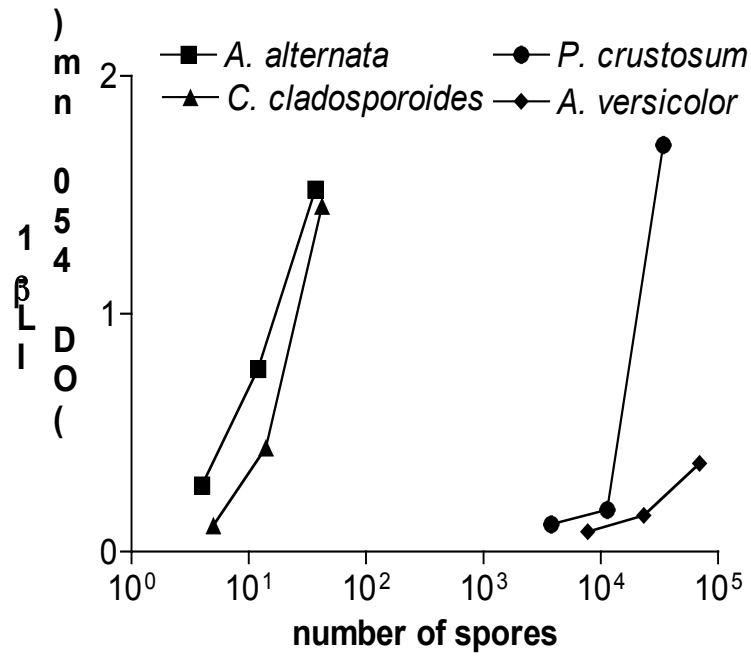
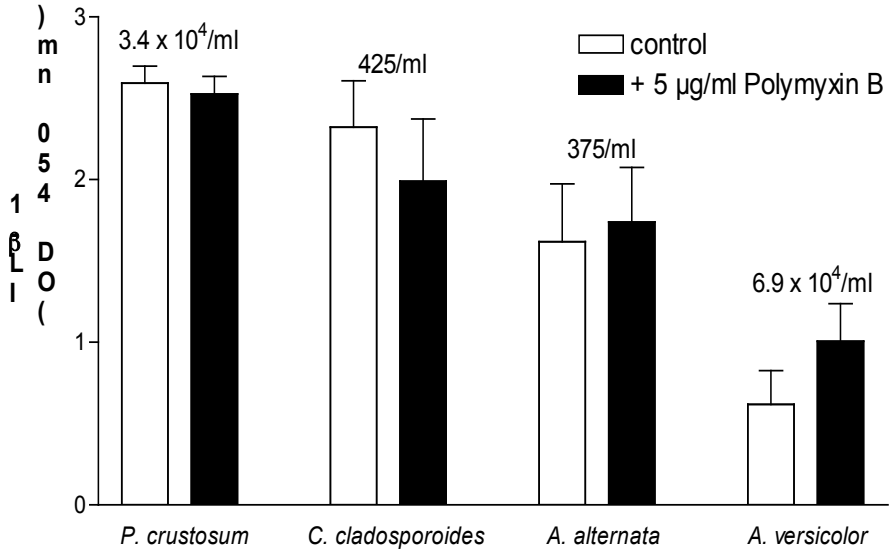


Figure: Fungal spores (Alternaria alternata, Cladosporium cladosporoides, Penicilium crustosum, Aspergillus versicolor) induce the release of IL-1□ by human whole blood.



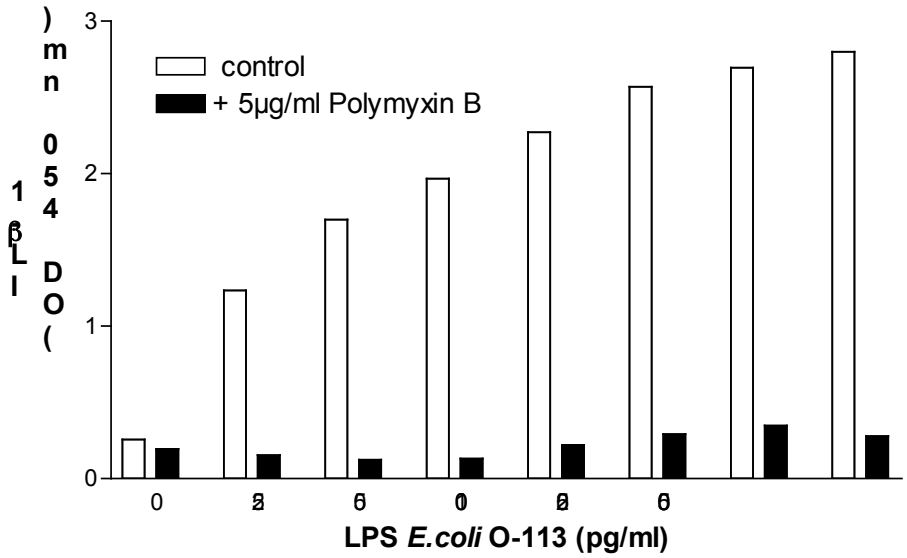


Figure 8: IL-1□ release in human whole blood in response to fungal spores (A) is not inhibited by 5 µg/ml polymyxin B, mean of 4 donors (±SEM), numbers above the bars indicate the spore counts employed. In contrast (B), the response to LPS is inhibited over a wide concentration range, mean of double values.

WB/IL-6
Carlin & Viitanen (In vitro pyrogenicity of diphtheria, tetanus and acelluar pertussis components of a trivalent vaccine, Vaccine 23, 3709-3715, 2005) report that IL6 release was triggered by toxoid deriving from Gram-positive bacteria *Corynebacterium diphtheriae* and highly purified lipoteichoic acid from Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*.

MM6/IL-6
Preliminary tests performed with other substances than described in the BRD have shown that the MM6/IL-6 assay is also reactive with non-endotoxin pyrogens. However, these data are not easily evaluated as there are no standards for pyrogens (other than endotoxin) available.

3. The test substances included in the BRDs are all parenteral pharmaceuticals. However, if the proposed test methods are intended as replacements for the current pyrogenicity test methods (i.e., rabbit pyrogen test; BET) shouldn't the validation studies also include other relevant test materials (e.g., medical devices, biologics, etc.)? Can you provide data for any of the proposed test methods tested with other relevant materials? If such data are not currently available, do you intend to generate data in a subsequent phase of testing?

As explained in the background information, by purpose the study has targeted areas of use of the rabbit test not the BET. Notably, the validation study did include human serum albumin and factor VIII preparations. Furthermore, supportive information is available for some of the tests (see also question 1). Since it is common practice to validate pyrogen tests for every given product and the restrictions in resources, the validation study itself could not cover additional substances.

WB/IL-1
In collaboration with the Brazilian National Control Authority, snake venom sera have been studied.

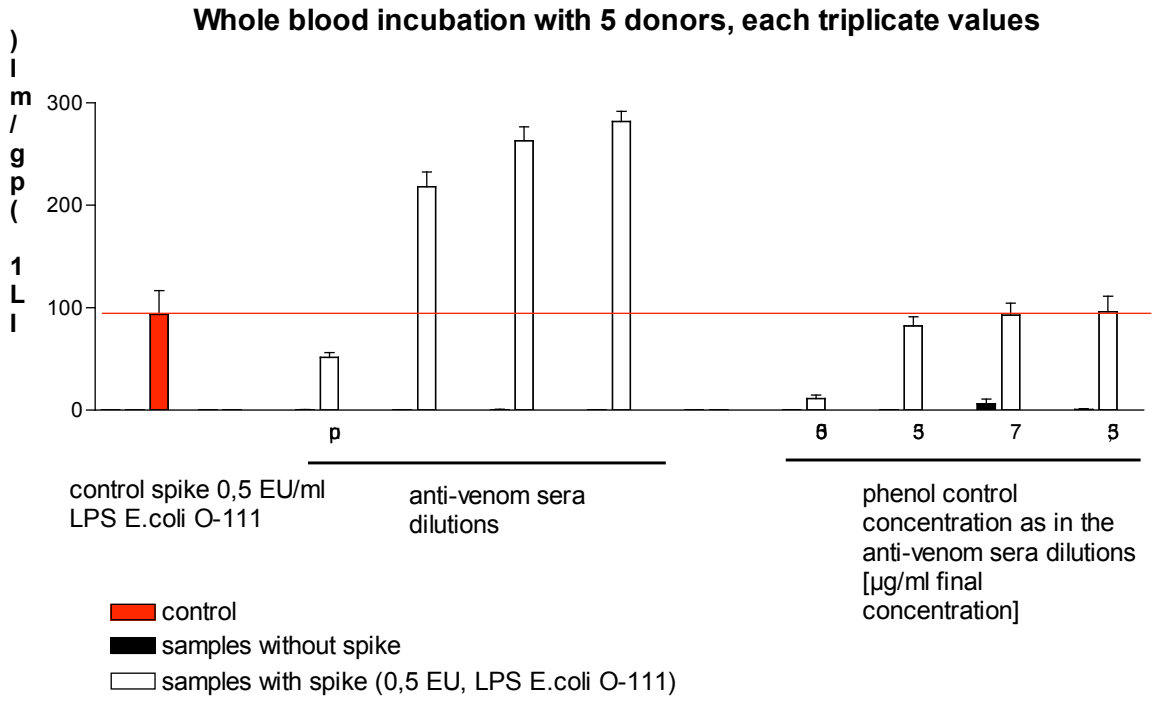


Figure: Spiked snake venom serum shows positive signal, though increased when compared to the control spike in saline.

Adaptation of the WB/IL-1 to biocompatible materials

Medical devices pose a particular problem since they cannot be examined directly with the abovementioned classical test systems. In order to be tested in the BET, an eluate has to be prepared, which is subsequently tested. This bears the risk to miss non-eluable, e.g. very hydrophobic, contaminations. Furthermore, the BET cannot detect non-endotoxin pyrogens. With the WBT, the material in question can be incubated directly and without any former treatment, provided, an adequate pyrogen-free control is provided. Apart from that their diversity with regard to size, form, material and form of application challenges biological assays demanding individual approaches. Products with direct (blood bags, needles) and indirect (swabs, gloves) contact to the blood circulation can have serious impact on the organism due to their permanent or transient contact with the blood stream or the lymph. A severe contact dermatitis due to endotoxin contaminations of surgical gloves was described in 1984 by Shmunes and Darby. After 8 pyrogenic reactions in altogether 69 patients undergoing heart catheterization, Kure et al. described endotoxin contaminations of extracts of the hospital's surgeon's latex gloves which evoked fever in the rabbit and could be successfully transmitted to cardiac catheters in 1982. Grötsch et al. were able to evoke fever reactions in rabbits with the eluate of gloves containing up to 2560 EU on their inside (Grötsch et al., 1992). Apart from that there are materials that are absorbed by the body, thus taking up any possible inherent pyrogenic materials, such as resorbable sewing materials. According to German legislation, implants are considered parenterals and therefore are drugs. For these products, according to the monograph "Parenterals" of European Pharmacopoeia, pyrogen testing is not required, but is suggested by several authors (Bohner at al., 1994, Grötsch and Eibach, 1990). The AAMI stated in 2001 that products with direct or indirect contact to the circulation system or the lymph or which interact systemically with the body should be tested for pyrogens (AAMI, 2001).

In order to judge a possible contamination, an eluate of the respective material must be either injected into the rabbit or used in the LAL. The alternative of transplanting the questionable

material directly into the rabbit is highly invasive causing possible reactions not associated with pyrogenic contaminations and is therefore questionable in its ethical and scientific implications. The obvious advantage of the IPT over the classical test methods is that the direct contact of the whole blood with the respective device does not require an eluate that allows only pyrogens to be detected which are not elutable for some reason. Additionally, unlike the LAL, the IPT detects all relevant pyrogens, not only endotoxin, in a species-specific manner. The basic principle consists of bringing the diluted human whole blood into direct contact with the surface of the material to be tested and incubate the blood for 10-24 hours at 37°C, like it has been established with the original method. Medical devices in this case included e.g. alginate microcapsules as carriers of drugs where the liquid material as well as the end product could be identified as pyrogen-free and an endotoxin control as well as a non-endotoxin spike was retrieved in an interference-free manner when compared to the same spikes in saline (see figure), dialysis membranes, dialysis fluids, bone substitute materials like tricalciumphospates, and implants such as hip joints. When testing filters, several, though not all, filter materials could be recognized in the WBT as pyrogenic or non-pyrogenic in an interference-free, dose-dependent manner.

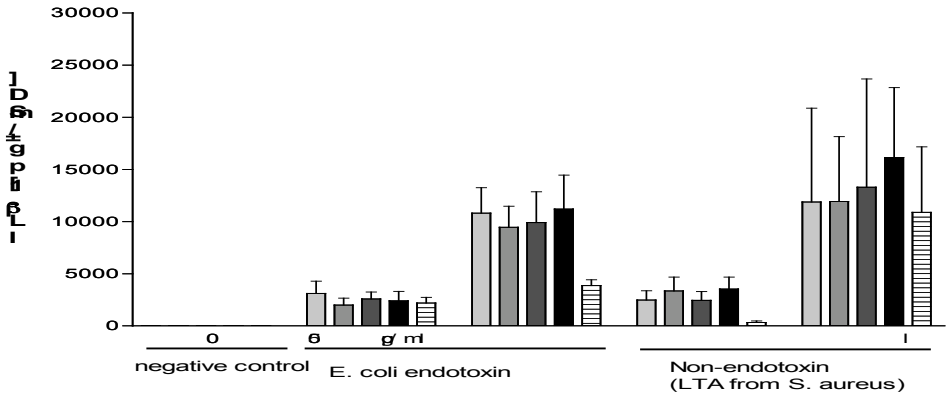


Figure: IL-1□ production of fresh blood of 4 donors upon stimulation with clean alginates and an artificial endotoxin/ non-endotoxin spike

Metallic and plastic surfaces

The testing for the inflammation inducing potential of implant surfaces for the judgment of biocompatibility is a relatively new field. In the early 1980s, it was noted that the monocyte is one of the first cells to arrive at an implant site and displays manifold functions (for review see Anderson, 1984; Ziats 1988). Its specific preference for rough and hydrophobic surfaces which develops after 7 days was termed rugophilia (Rich and Harris, 1981). In 2002, Soskolne et al. documented the adhesion and secretion of TNF-□ of monocytes on titanium surfaces that increases with increasing degrees of roughness; a finding that was later confirmed by Refai et al., 2004 who extended the study of Soskolne to IL-1□ and IL-6 as well. The authors found an enhanced secretion of all three proinflammatory cytokines towards a low LPS dose on the roughest titanium surface with no IL-1□ and IL-6 secretion of unstimulated cells. In contrast, TNF-□ secretion was elevated on this surface, even without LPS stimulus. Another titanium surface that had been polished tended to diminish IL-1□ and IL-6 secretion after LPS stimulation (Refai et al., 2004). The role of cytokine production of the monocytes/macrophages in the early stages of implant insertion are until now poorly understood. The fact that obviously some materials are capable of modulating the cytokine response makes it difficult to distinguish a genuine pyrogenic contamination from an unspecific activation and poses the problem of adequate negative controls. For this purpose, in a master-thesis by Kullmann in 2002, a model was developed for the testing of metallic or plastic surfaces. The study showed that pyrogenic contaminations on surfaces could be reliably removed only when heated for 5h at 300° C with no differences in varying surface

structures (rough or smooth). This applied to titanium, titanium alloy (TiAl6V4) and steel material for implants. The blood was incubated directly in a depyrogenized microtiter plate with contact to the surface to be tested. Artificial contaminations could be recognized in a dose-dependent manner and removed by different washing/heating procedures. With this procedure, safety testing of medical devices with the IPT can be performed, with the possibility of testing the material itself without an eluate and an adequately depyrogenized negative control.

Aneurysm clips made of titanium

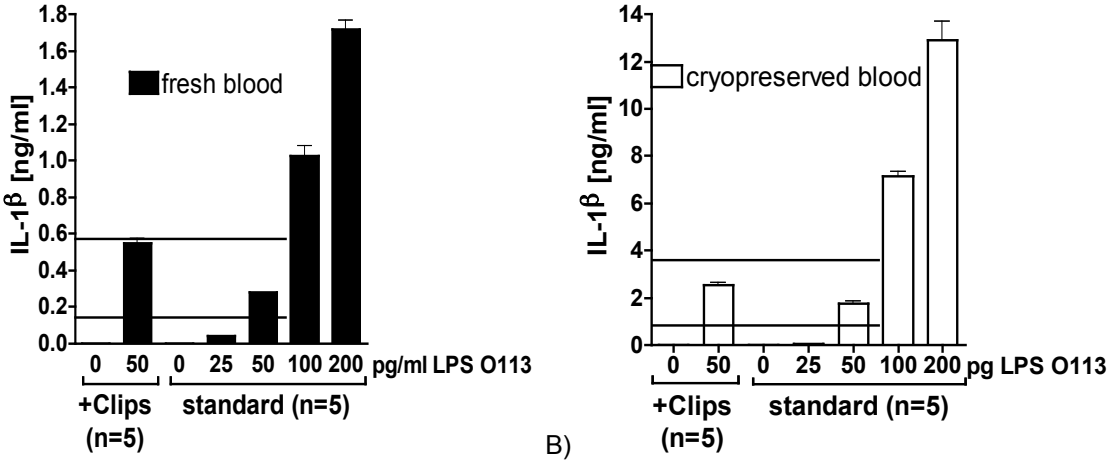


Figure: Comparison of the use of fresh and cryopreserved human blood for pyrogen detection on titanium clips.

5 clips each were incubated with 100 µl saline or LPS (50 pg/ml) diluted in 100 µl incubation medium. Then 100 µl fresh blood (panel A) or cryopreserved blood (panel B) were added and IL-1□ release was measured by ELISA. Data are given as mean ±SD or rather as median. The horizontal lines mark 50% and 200% of the IL-1□ release in response to 50 pg/ml LPS O113 in the absence of a clip.

A) Titanium (aneurysm clips)

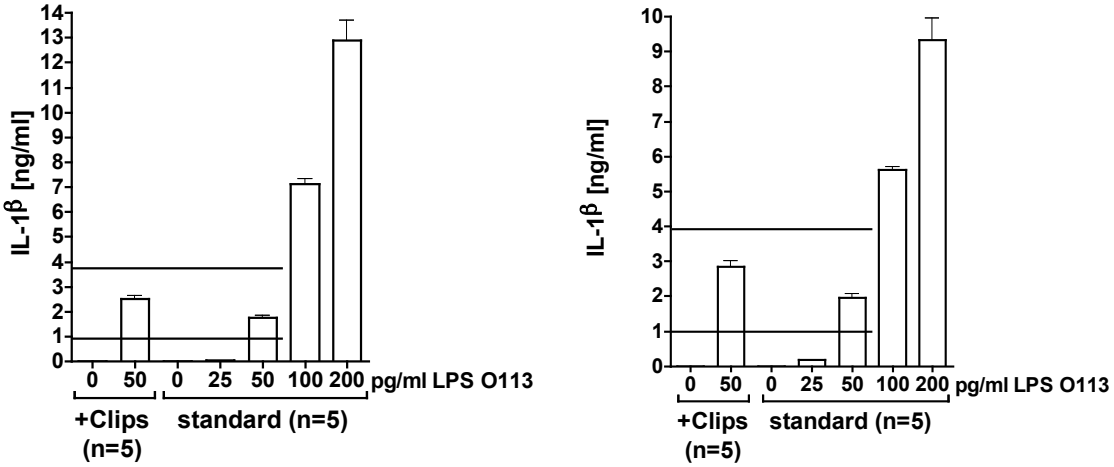


Figure: IPT with aneurysm clips made of titanium

Incubations were performed with cryopreserved blood and IL-1□ was measured by ELISA. Data are given as mean ±SD or rather as median.

material	device	effects on IPT
Chirulen 1020		none
Chirulen 1050		none
metallocene-PE	human blood bags	none
micoporous surface on titanium	chips	none
nickel silver 2.0441	bar stock	increasing
PA6.6.	bar stock	none
PEEK	bar stock	increasing
polyamide coat	ablation catheter	increasing
polyamide coat	balloon catheter	increasing
polyethylene coat	heartwire	none
polypropylene with titanium coating	mesh-implant TiMESH	none
polyurethane Elastollan	pellets	none
polyurethane film	boxes for clips	none (fresh blood)
polyurethane film	boxes for clips	decreasing (cryo blood)
polyurethane foam with polytetrafluoroethylene film	synthetic skin substitute	decreasing
POM	bar stock	increasing
PPSU	bar stock	increasing
stainless steel 1.4021	bar stock	increasing
stainless steel 1.4306	bar stock	increasing
stainless steel 1.4401	bar stock	increasing
stainless steel 1.4542	bar stock	increasing
Titanium	aneurysm clips	none
Titanium 3.7165	bar stock	increasing

Table: Summary of the results of influences of the tested materials on the IPT

Dialysis

In the US, the number of patients receiving dialysis has almost tripled from 63.000 to 170.000 between 1982 and 1992 (Bland, 1995). In the same time, the percentage of dialysis centers that reprocess hemodialyzers for reuse has increased from 19 to 72%. Pyrogenic reactions of hemodialysis (HD) patients at the end of a session were first associated with high bacterial and endotoxin levels by Raij et al., 1973 and Favero et al., 1974.

Since then, possible contaminations could be attributed to the pure water (Klein et al., 1990, Pegues et al., 1992, Kulander et al., 1993, Bambauer et al., 1994) to the machines, especially to areas with low circulation or dead spaces which serve as a reservoir for bacteria (Phillips et al., 1994), the filter materials (Schouten et al., 2000) and bicarbonate concentrates (Pegues, et al., 1992).

In 1993, the AAMI (Association for the Advancement of Medical Instrumentation) released recommendations for the quality of treated water and dialysate which restricted the content of heterotrophic bacteria to 200 and 2000 cfu/ml, respectively. Studies in Germany (Bambauer et al., 1994), Greece (Arvanitidou et al., 1998), the USA (Klein et al., 1990), and Canada (Laurence and Lapierre, 1995) revealed that even these moderate standards are not met, which is even more critical considering that a patient with chronic renal failure receives up to 400l of dialysis fluid a week. For example, in the US, 53% of the treated water did not comply with AAMI standards (Germany 17.8%, Greece 6.4%). According to the study of Bamberger et al., in Germany, 12.2% of the water samples contained 5 EU/ml or more with dialysate contaminations of up to 487 EU/ml. Pseudomonas was found in the water of 73% of the centers (dialysate 90%). Cocci (Micrococci, Staphylococci and Streptococci) were found in the dialysate of 83, 70, and 10% of the centers, respectively, indicating the importance of Gram-positive contaminations. Nakagawa et al., 2002, did an extensive study of the detection of non-endotoxin pyrogens in the whole blood assay and a cell line in comparison to the rabbit. That this might indeed be crucial for judging the pyrogenic load for a dialysis patient was assessed by Marion-Ferey et al., who tested scrapings of bacterial

biofilms in dialysis tubes and found a 20fold higher response in the IPT than in the LAL (Marion-Ferey et al., 2005). Petri et al., 2000, also reported better recognition of Gram-positive and Gram-negative stimuli in the WBT when compared to the LAL.

The passage through dialysis filters of cytokine-inducing substances, not only endotoxins, but exotoxins and peptidoglycans as well, have been demonstrated (Evans and Holmes, 1991, Lonnemann et al., 1992, Urena et al., 1992, Tsuchida et al., 1997). That this indeed can pose a severe threat to patient health has been the result of extensive research over the past years (Schindler et al., 1996, Lonnemann 2000). More than one clinical pyrogenic reaction per year occurred in the US in about 20% of all dialysis centers (Tokars et al., 1991).

A majority of authors found elevated cytokine levels in unstimulated patient blood or an enhanced reactivity of the monocytes of uremic HD patients towards low doses of LPS when compared to a healthy group, on the mRNA level (Pertosa et al., 1993, Schindler et al., 1993, Girndt et al., 1995), on the protein level (Haeffner-Cavaillon et al., 1989, Girndt et al., 1995) as well as recently in vitro employing the whole blood test (Vaslaki et al., 2000, Canaud et al., 2001). Girndt et al. could associate the higher IL-6 production in vitro with elevated in vivo IL-6 serum levels and showed an impeded immune response towards vaccination in these patients. That IL-1□ plays an important role in the pathogenesis of HD related complications were reviewed by Dinarello in 1988. Elevated intracellular IL-1□ in unstimulated patient samples and a reduced reactivity to a high LPS dose (2µg/ml) was found by Blumenstein et al., 1988. Donati et al. showed in 1997 elevated unstimulated plasma levels of IL-1□ and IL-1ra as well as a strongly increased proportion of monocytes expressing the IL-1 receptor (8.7± 1.9% in healthy subjects compared to 31.5 ± 3.5% in HD patients).

The long-term consequences of chronically increased cytokine levels seem to be even more relevant. Even if cytokines are not the cause of amyloidosis, they might aggravate the process. In 1991, Baz et al showed that the use of ultrapure water delays the onset of the carpal tunnel syndrome. The group of Schwalbe showed in 1997 in a retrospective study that the incidence of amyloidosis decreased between 1988 and 1996 along with the disappearance of a water softener known to promote bacterial growth and the introduction of reverse osmosis, a very effective method for purifying water. A connection between other phenomena such as malnutrition, poor immune responses and high incidence of malignant tumors in long-term HD patients and cytokine production has yet to be firmly established.

A possible method for testing dialysis fluids in the WBT used varying percentages of diluents and samples (unpublished results of the developing laboratory). While the classic WBT protocol involves 1 ml of 0.9% saline, this was replaced by 1 ml of pyrogen free water in order to compensate the high electrolyte content of e.g. bicarbonate fluid. For the testing of water, a reverse protocol was employed, using 1 ml of the water to be tested in order to lower the detection limit of the test (usually 0.25 EU/ml) and instead of sample 100 µl of 40% sterile saline (Table).

Basic protocol	Modified protocol for electrolyte solutions	Modified protocol for dialysis water
1000 µl of 0.9% saline	1000 µl of pyrogen-free water	1000 µl dialysis water
100 µl sample	100 µl electrolyte solution	100 µl 40% saline
100 µl blood	100 µl blood	100 µl blood

Table: Possible modification of the WBT standard protocol for the testing of dialysis fluids

Notably, a variant of the WBT, termed AWIPT (see responses to questions 4 & 5), offers the opportunity via endotoxin extraction and accumulation to lower the limit of detection and remove interfering substances, both relevant for dialysis fluids.

Lipidic formulations

Since January 2004, the testing of so-called small volume parenterals (< 15ml) has been made obligatory by European Pharmacopoeia. This concerns many formulations that have not been subjected to pyrogen testing before such as vitamin preparations and steroids. Many of these are applied intramuscularly or subcutaneously and therefore not necessarily have a hydrophilic nature. This poses a completely new challenge to all methods of pyrogen testing, since a lipophilic substance cannot be injected intravenously into the rabbit and will, on the other hand, influence the OD measured in the BET due to the formation of miniscule oil drops. Furthermore, the pyrogenic portion of the LPS, the lipid A (for review see Rietschel et al., 1993) has been reported to be masked by lipoproteins (Emancipator et al., 1992) and lipophilic parenterals (Paulssen and Michaelsen, 1984) in the LAL. Therefore, the WBT procedure was adapted to suit lipophilic substances (Schindler S., submitted). As a first step, interference-free oils such as sesame oil were identified by comparing an LPS dose response curve in these oils with a similar curve done in saline. Surprisingly, many oils (sesame oil, peanut oil, paraffin, miglyol) were interference-free, while others, and especially drug-containing end products, interfered strongly by suppressing the endotoxin stimulus added. Oils that tested interference-free were then used as diluents for interfering end products. It was possible to dilute the interference to non-detectable limits with full recovery of an artificial endotoxin spike. From this minimum valid dilution a possibly detectable endotoxin concentration could be calculated, which was 20 EU/ml for the respective end products. Since these products are applied at a very small volume (1ml per person) a relatively high endotoxin contamination can be tolerated which will predictably not pose a health hazard for the recipient. The established protocol leaves a broad safety margin, especially since the criteria for intravenous drugs were applied (Schindler et al., submitted).

Conclusion: The WBT using fresh as well as cryopreserved blood is a useful and reliable tool for several aspects of pyrogen detection. Not only does it detect a wide spectrum of possible fever-inducing substances, but also its robustness makes it available for such different aspects as the testing of solid substances, (immuno-) toxic drugs, air quality, and biologicals.

MM6/IL-6
Testing of medical devices, immunoglobulins and antibodies has not been performed and additional testing is not foreseen in the developing laboratory in the near future.
Testing of vaccines using a MM6/IL-6 assay has been very limited so far (Carlin and Viitanen, Pharmeuropa Vol 15, no 3, 2003, page 418-423). Results with the multivalent vaccine Infanrix suggest that (some of) the components inhibit IL-6 production by MM6-cells (Infanrix interfered also with the BET assay). Preliminary experiments also indicate that interference should be assessed.

4. There are well-known limitations of the BET (e.g., proteins that interfere with LAL, chemical extracts from medical devices). What are the specific limitations of the proposed test methods? Do certain physicochemical properties interfere with these assays (e.g., pH, osmolarity, protein content) and, if so, what are they and how do they encumber these test methods?

In general, the novel tests are not suitable for drugs that interfere with the cytokine readout. This applies to all drugs that either raise or diminish the signal. In order to judge this positive or negative interference, the Limulus criterion of a 50-200% retrieval was chosen, comparing the spiked sample to the reference control value in saline. The SOPs of all tests include sections on testing for interference. The available data show that interference testing is necessary and acceptance criteria need to be established for the individual pharmaceutical products.

The toxicity and pyrogenicity in man is well known for LPS but only from E. coli. For other endotoxins e.g. from Pseudomonas or Salmonella we know that they can be 1000 times less or 10 times more toxic. About combinations of endotoxins or even combinations of endotoxins and non-endotoxin pyrogens there is very limited experience. Moreover there is some concern about potentiation of the activity of low endotoxin contamination together with a usually non-pyrogenic contamination like glucans or pepdidoglycan (Traub S, von Aulock S, Hartung T and Hermann C. MDP and other muropeptides – direct and synergistic effects on the immune system. J Endotox Res 2005, in press.; Traub S, Kubasch N, Morath S, Kresse M, Hartung T, Schmidt RR and Hermann C. Structural requirements of synthetic muropeptides to synergise with LPS in cytokine induction. J. Biol. Chem. 2004, 279:8694-8700.). The conclusion is that every drug product has to be validated individually and the acceptance criteria have to be defined very carefully (together with statisticians and physicians). The testing for interference is described in all SOPs of the novel tests.

The individual tests differ slightly with regard to the dilution of the test sample (from 1:1 to 1:12), which results in some differences with regard to the limit of detection but also how much they are prone to interference with the products. The WBT tests are extremely robust due to the buffer capacity of human serum (e.g. allowing samples of pH 1 or pH 14 to be tested) and the strong dilution (1:12).

WB/IL-1
A selection of drugs all known to interfere with the rabbit pyrogen test and in part also with the Limulus test, i.e. taxol, was used to test the efficiency of pyrogen detection by WB/IL-1 and the newly developed AWIPT. The AWIPT (Absorb and Wash In vitro Pyrogen Test) is a new development that enables all drugs interfering with the WBT to be tested. Endotoxins are extracted by endotoxin-binding beads, which are after a wash step that takes out the interfering materials, exposed to the WBT. This makes use of the unique property of the WBT to allow endotoxin determination on solid materials. The AWIPT allowed spike recovery at a dilution at least a factor 3 less than in WBT for all drugs except for gentamicin spiked with 25 pg/ml LPS where the recovery was the same in both tests. The safety margin required to exclude relevant pyrogenic Intentional contaminations with lipopolysaccharide were retrieved from the chemotherapeutic agents paclitaxel, cisplatin and liposomal daunorubicin, the antibiotic gentamicin, the antifungal agent liposomal amphotericin B, and the corticosteroid prednisolone at lower dilutions than in the standard in vitro pyrogen test.

In this study, such problematic substances were addressed using macroporous acrylic beads decorated with immobilized human serum albumin (HSA). The material was originally developed as an extracorporeal endotoxin-adsorbing matrix to treat endotoxemia and sepsis. Albumin is a universal carrier of lipophilic substances like fatty acids, bilirubin and hormones in plasma via hydrophobic interactions in a molar ratio of up to 10:1. This test represents a promising new approach to test interfering drugs or drugs containing interfering additives for pyrogenic contaminations, thus improving the safety level of the drugs.

	WBT	AWIPT	WBT	AWIPT
	Dilution at recovery of spike (25 pg/ml LPS)		Dilution at recovery of spike (50 pg/ml LPS)	
paclitaxel	100	10	100	31
gentamicin	31	31	100	31
Cisplatin	100	10	>316	31
prednisolon	> 316	10	>316	31
liposomoal amphotericin B	10	3	31	10
liposomal daunorubicin	>316	31	>316	100

Table: Minimum dilutions of complex therapeutics at recovery of LPS spike.
Drugs were diluted in series and spiked with 25 pg/ml or 50 pg/ml LPS. Recovery of spike, defined as cytokine release at levels between 50% and 200% of those induced by the same concentration of LPS in the absence of the drug, was achieved at the given dilutions.
Additionally, the recognition of LPS as well as LTA can be improved by immobilizing it on a surface. Therefore, the AWIPT cannot only be used to enhance LPS detection, but that of LTA as well.

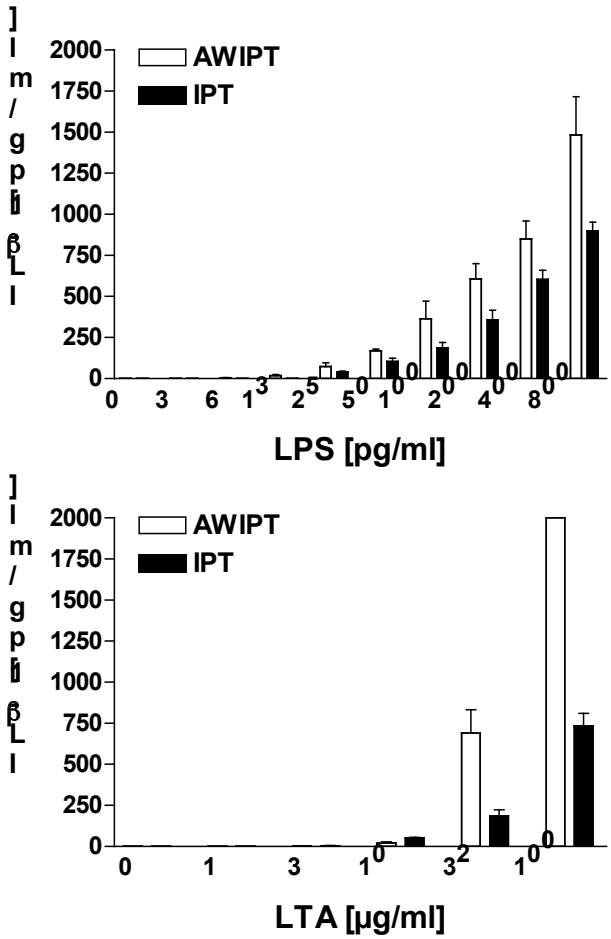


Figure: Comparison of IL-1β response to LPS and LTA in IPT and AWIPT
100 µl samples of 3 to 800 pg/ml LPS or 1 to 100 µg/ml LTA were employed in parallel in IPT and AWIPT. IL-1β release was measured by ELISA. Data represent means ± SD of triplicates from one blood donor.

5. The current bacterial endotoxin test requires standardization of the Limulus amoebocyte lysate (LAL). How would the cellular components of the proposed test methods (i.e., whole blood, PBMCs, cultured monocytoid cell line) be standardized?

WB/IL-1 & WB/IL-6
Work in several hundred blood donors has shown that the threshold of cytokine induction and the levels of cytokine released are sufficiently conserved among individual donors. Extreme reagibilities are very rare and are controlled by the controls employed. Since the donor's individual response curve to endotoxin serves as calibrator in each measurement, any difference in

responsiveness is controlled for. The availability of standardized kit versions including control reference endotoxins further contributes to the standardization of the test.
Further standardization is achieved by cryoconservation of blood as demonstrated in the validation. A blood donation as for transfusion purposes (500ml) by five pooled donors would suffice for up to 25.000 measurements. Shelf lives of more than one year have been established at -80 degree and liquid nitrogen, respectively. Certified cryoblood produced according to GMP and ISO standards is already commercially available.

PBMC/IL-6
The method with PBMC/IL-6 sees 2 types of test: First of all, an investigation for interferences of the test substance with the test system and the readout system has to be performed and then the highest test concentration not showing interferences is determined. The first type of test is to calibrate each donor response on an individual calibration curve produced with the standard endotoxin. The donor response is then defined in terms of "Endotoxin Equivalents" and the product specification is equal to the endotoxin limit concentration (ELC). The standardization goes through multiple donor testing and setting of adequate acceptance criteria. The second type of test (as described in the SOP) is to test against a "clean" reference preparation of the same product. The standardization is again assured by testing of PBMC coming from different donors.

MM6/IL-6
In our opinion the proper procedure to standardize the MM6/IL-6 is equivalent with the procedure described for PBMC/IL-6. Interference of a drug product with the cells and the readout system should be tested with a number of different batches of the same product (known to be pyrogen free). The highest test concentration not showing interference (or an acceptable level of interference) is determined. This concentration should be applied for testing suspect products. The suspect product should always be tested against a “clean” standard reference preparation of the same product.

6. The prediction model described in the BRDs is based on a pyrogen threshold concentration of 0.5 EU/mL. While this level of detection would indeed suffice for many parenteral drugs and medical devices, the endotoxin limit set by the U.S. Food and Drug Administration for intrathecal drugs and devices that contact cerebrospinal fluid is 0.06 EU/mL. Do you have data to support the use of the proposed test methods for discriminating an endotoxin threshold lower than 0.5 EU/mL?

The immune system of all organisms reacts extremely sensitively to bacteria recognizing conserved structures often termed endotoxins. Here, man, rabbit and horseshoe crab do not differ very much. The thresholds of reaction are remarkably conserved in the low picogramme range or about 0.1 to 1 bacteria per immune cell. The precise set-up of the test is determining the limit of detection. For the purpose of predicting the rabbit response, tests were adjusted to a threshold of 0.5 EU/ml. Given a routine dilution of the samples of up to 1:12 and measurable signals also at endotoxin concentrations lower than the 0.5 EU threshold demonstrates that the sensitivity of the systems has not been fully exploited.

WB/IL-1
The test has been modified to include adsorption of endotoxin to beads (termed AWIPT, see above). This offers the possibility, by concentrating the LPS on its surface and enhancing the reactivity of the monocytes, to detect as little as 0.0001 EU/ml (see Figure)

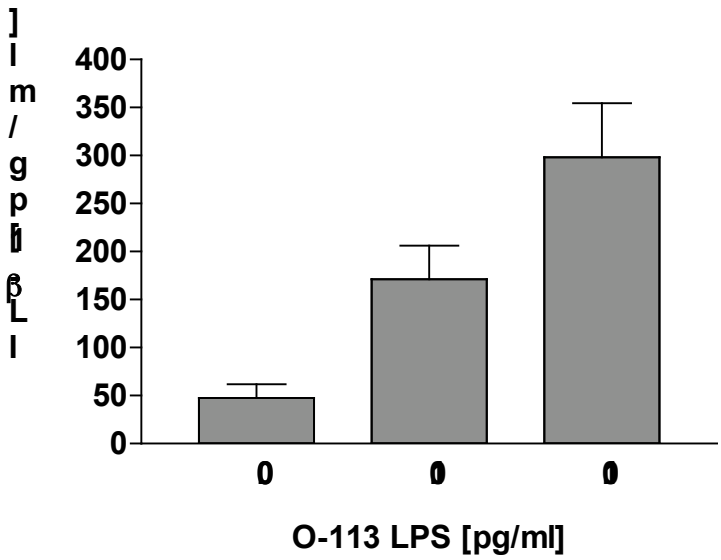


Figure: Limit of detection in the AWIPT

MM6/IL-6
During the development of the assay it was chosen to settle for a threshold concentration of 0.5 EU/ml. However, the sensitivity of the MM6/IL-6 is as low as 0.1 EU/ml in most experiments. Aiming at a threshold level of 0.06 EU/ml will challenge the assay.

PBMC/IL-6
The sensitivity against standard endotoxin of the test with PBMC/IL-6 is comparable to the BET. The detection limit is about 0.01 EU/ml.

Comments and suggestions relevant to all BRDs

* ***A list of definitions would be useful.***

Such a list will be included.

* ***There appear to be different designations for the cells/tests in the BRDs, in document Stp-HPTVv04, Comparison and Validation of Novel Pyrogen Tests Based on the Human Fever Reaction. Trial Plan, and the file names and nomenclature of the tests in the SOPs. The cell/test nomenclatures should be standardized to avoid confusion. Examples of various designations used are: PBMC-IL6; PBMC; WB-IL6; WB/IL-6; WB-IL1; cryo WB-IL1; WB-CRYO/IL-1; MM6/IL6; MM6.***

This will be amended as far as it is not part of historic documents, where tests were termed differently.

* ***Sections 4.1 and 4.6 comment on theoretical assumptions of sensitivity and specificity, and cite reference [10]. There is no reference [10] in the BRDs; the citation, if relevant, should be provided in the format used for the BRDs.***

This has been corrected.

* **The BRDs mention that the SOPs for the different tests are in Section 13.1. However, Section 13.1 only references the SOP; copies of the SOPs are included in Section 15 of each BRD.**

The BRDs have been revised and the method protocols and trial plans are now included in Appendix A.

* ***In all the submitted studies, the accuracy of the test is being measured using bacterial endotoxin (LPS) in all test samples; presumably only the vehicle is changed. However, one of the claimed advantages for this test over the BET is that it is capable of detecting non-LPS pyrogens, whereas the BET cannot. There are no test results from the non-LPS pyrogens referred to in the Rationale. In the absence of additional data on other pyrogens, it cannot necessarily be assumed that the tests would be relevant for non-LPS pyrogens.***

See 2)

* ***The conclusion in Section 6.4 that the test is applicable to "most classes of medicinal products> needs expansion and clarification. First, the product classes to which the test is not applicable should be identified; there appears to be no information in the BRD at this point. Secondly, the statement should be clarified to state that the test is applicable to the detection of LPS. No evidence has been presented with respect to other classes of pyrogens.***

Rabbit pyrogen testing as the reference method has been substituted by large extent with the BET, which is a mere endotoxin test. The remaining rabbit testing is due to interference of test materials with the BET and not due to its limitations to endotoxin. A novel substitute for the rabbit test should be evaluated on the same basis. Since non-endotoxin pyrogens have not been internationally agreed and made available as reference materials, a formal validation is not possible. The supportive information that the in vitro pyrogen tests cover in fact some of the presumed non-endotoxin pyrogens represent a characteristic in favour of these tests compared to the BET.

* ***There is a comment in the BRDs (Section 4.1) regarding historical data from rabbit tests, yet the sensitivity, specificity, and concordance values presented are not related to the in vivo data (i.e., Section 6.2, Section 6.3). It is not clear how these values could be generated without relating to rabbit or human test results on the same samples.***

On the basis of the determined rabbit fever threshold (Hoffmann S, Luderitz-Puchel U, Montag-Lessing U and Hartung T. Optimisation of pyrogen testing in parenterals according to different pharmacopoeias by probabilistic modelling, J. Endotoxin Res. 2005, 11:25-31), it was possible to model the performance of these rabbits, when testing the samples of the validation study assuming no additional interference of the samples. The sensitivity of the rabbit pyrogen test is 57.9% and the theoretical specificity of the rabbit pyrogen test is 88.3%. Unfortunately these numbers have not been correctly reported in the BRD, in sections 4.2 and 4.6., where the last sentence reads " .. sensitivity of the rabbit pyrogen test is 75.04% and the theoretical specificity of the rabbit pyrogen test is 95.80%.

* ***There appears to be little relationship among the articles cited in each BRD, the list of references in Section 12 of each BRD, the articles included in Section 15 of each BRD, and the articles supplied in electronic format. This could be a problem if a reviewer wanted to read a referenced article. The reference citations in the text, the list of references in Section 12, and the copies of the references in Section 15 need to be coordinated. These discrepancies are presented in different levels of detail in the following BRD assessments.***

The references have been corrected and hardcopies of a number of publications are included in Appendix B.

* ***There are statements in Section 9.3 of all the BRDs that compare the performances of different tests, but data are not presented. The data are in the referenced Hoffmann publication, which was submitted on a CD file, but not in hard copy. It would assist in the assessment of these assays if the Hoffmann article could be appended to the individual reports, and if summary performance tables were included in each report to support the brief verbal description of test performance that appears in Section 9.3.***

Added as requested.

* ***It would be helpful if the articles in Section 15 were in alphabetical order.***

The hardcopies of a number of articles are in Appendix B.

* **It would be helpful to the reviewers to have a table comparing the strengths and weaknesses, if any, of the assays.**

See table 9.3.2 in Section 9 of the BRDs

* ***Some, if not all, of these tests are patented. The patented tests and procedures should be identified. The Sections on test method transferability (Section 11.1) and cost (Section 11.3) should address the availability, licensing fees and licensing agreements, if any, of these tests.***

This is now mentioned in the individual BRDs.

Comments on the individual BRDs

Comments relevant to the Human PBMC/IL-6 In Vitro Pyrogen Test

* *Section 1.1.2. The results of the cited FDA peer review have not been summarized or provided.*

The following information is now included: The PBMC/IL-6 test developed by Novartis and Baxter Healthcare has been subjected to a rigorous peer-review by the US FDA and approved as an end-product release test (New Drug Application Number 16-267/S-037 approved on April 24, 2002).

* *Section 2.1. The file reference in Section 2.1 should name the specific file because there are two PBMC SOP files, and there is no information provided as to which SOP file is referred to in the BRD.*

This has been amended in Section 2.1 and Section 13 (catch-up validation of PBMC CRYO IL/6) and Appendix A includes both method protocols.

* *Section 2.3. Define LAL*

LAL = Limulus amoebocyte lysate

Please note that the term "LAL" has been replaced in the 5 BRDs with the more general term BET (= Bacterial Endotoxin test), which is based on the use of Limulus amboecyte lysate.

* *Section 2.4. The data presented here were obtained using a Novartis-developed IL-6 ELISA assay, and this section states that any commercial IL-6 ELISA kit will have to be validated for this pyrogen test. Unless the Novartis assay will be publicly available, non-Novartis users (who, presumably, the test is designed for) will have to go through a separate validation of this assay.*

Any human IL-6 ELISA can be used provided International Standard (IS) for IL-6 (or an IL-6 standard calibrated against the IS) is used as the assay calibrant. In addition, it would be necessary to demonstrate that the requirements of test controls are met and no interference with the test substances occurs.

For the two other methods with IL-6 release as an endpoint used either the Novartis IL-6 ELISA (WB/IL-6) or a commercial kit (MM6/IL-6).

* *Table 3.3.1. Define > "> notional ELC> "> .*

notional ELC = endotoxin limit concentration set by the European Pharmacopoeia monograph (or other guidelines) for a given product. The term is explained in each of the five BRDs.

* *Section 12. There are a number of discrepancies among the cited articles, the bibliography, and the provided references. Many of the publications listed here do not correspond with those cited in the submission or those included as hard copies in Section 15.*

This was resolved. Cited articles are listed in Section 12, articles in bold are attached as hardcopies in newly created Appendix B. Appendix B includes the same set of publications for the 5 BRDs.

Comments Relevant to the Human Whole Blood/IL-1 In Vitro Pyrogen Test

* There are no comments specific to this test method. There are reference and citation problems similar to those identified in Sections 2.6.2 and 2.6.6.

This was resolved. Cited articles are listed in Section 12, articles in bold are attached as hardcopies in newly created Appendix B. Appendix B includes the same set of publications for the 5 BRDs.

Comments relevant to the Human Whole Blood/IL-1 In Vitro Pyrogen Test: Application of cryopreserved human whole blood

* *Although this is a test method that uses cryopreserved blood, the SOP in Section 15 does not address cryopreservation.*

Unfortunately, there was a mistake with the first submission, the correct protocol is now given in Appendix A of BRD CRYO WB/IL-1. In addition, the article Schindler et al, 2004 in appendix B deals with cryopreservation.

* *Section 2.5. An abbreviated validation study was performed. The validation study of this method appears to consist only of a comparison of the results from using cryopreserved blood with the results from the same test (WB/IL-1) using fresh blood. Therefore, the statements on (intra-laboratory) reproducibility should be removed from Section 3.1 and Section 5 because Section 2.5 and the data in Section 5 indicate that reproducibility was not examined.*

Data of on intralaboratory reproducibility are included in Appendix D of the BRD CRYO WB/IL-1.

* *There are reference and citation problems similar to those identified in Sections 2.6.2 and 2.6.6.*

This was resolved. Cited articles are listed in Section 12, articles in bold are attached as hardcopies in newly created Appendix B. Appendix B includes the same set of publications for the 5 BRDs.

Comments Relevant to the Human Whole Blood/IL-6 In Vitro Pyrogen Test

* *Section 2.4. The data presented here were obtained using a Novartis-developed IL-6 ELISA assay, and this section states that any commercial IL-6 ELISA kit will have to be validated for this pyrogen test. Unless the Novartis assay will be publicly available, non-Novartis users (who, presumably, the test is designed for) will have to go through a separate validation of this assay.*

Any human IL-6 ELISA can be used provided International Standard (IS) for IL-6 (or an IL-6 standard calibrated against the IS) is used as the assay calibrant. In addition, it would be necessary to demonstrate that the requirements of test controls are met and no interference with the test substances occurs. For the two other methods with IL-6 release as an endpoint used either the Novartis IL-6 ELISA (PBMC/IL-6) or a commercial kit (MM6/IL-6; see below).

* *There are reference and citation problems similar to those identified in Sections 2.6.2 and 2.6.6.*

This was resolved. Cited articles are listed in Section 12, articles in bold are attached as hardcopies in newly created Appendix B. Appendix B includes the same set of publications for the 5 BRDs.

Comments Relevant to An Alternative In Vitro Pyrogen Test Using the Human Monocytoid Cell Line MONO MAC-6 (MM6)

* *Section 2.6. This suggests that only the Novartis IL-6 ELISA assay is usable because other ELISAs were not repeatable, and therefore could not be used. This aspect should be expanded upon because it suggests that users of the MM6 test will be limited in the ELISA preparations they can use or, alternatively, have to validate the test using other ELISAs.*

The initial protocol allowed the use of various kinds of IL-6 ELISAs, however, due to their sub-optimal repeatability their use was restricted to the two IL-6 ELISAs now indicated in the protocol (the in-house Novartis IL-6 ELISA and the CLB Human IL-6 ELISA kit). Both IL-6 ELISAs use the same monoclonal antibody for IL-6 detection. It should be noted that these ELISAs may be substituted with other validated IL-6 specific ELISAs, in which the International Standard (IS) for IL-6 (or an IL-6 standard calibrated against the IS) is used as the assay calibrant.

* *Section 2.4. The cell line that was used for this validation study is available only from a particular laboratory at the University of Munich, Germany, which will supply the cells to all who request them, or from a Master Cell Bank and a Working Cell Bank at the NIBSC (in Germany) (see also, SOP Section 6.1). Another source of cells is the German DSMZ. It is stated here that the performance of the DSMZ-source cells have not been compared with the NIBSC cells used in this validation study, and there is no mention of whether the performance of the NIBSC cells was compared to the Univ. of Munich cells. All cell sources are listed under the heading of > "> Proprietary Components.> "> The BRD should address whether these cells are proprietary, and if there will be a one-time cost or licensing fee, or a licensing agreement, before they can be shipped to a testing laboratory. The sponsor should ensure that the various cell lines are interchangeable in the assay; otherwise, there will be only a single source for the cells. Alternatively, the validated cell line should be more widely distributed so that there would be less likelihood of loss, and so that users outside of Europe will have more easy access to them. Therefore, it may be reasonable to recommend that the sponsor ensure the continued availability of a cell line or lines whose performance of the test is well documented before ICCVAM invests time and resources on the evaluation of this test.*

The performance of the cells obtained from NIBSC (UK) was not compared to cells directly obtained from the University of Munich, Germany or the DSMZ.

The MM6 cell line was established by Prof. H.W.L. Ziegler-Heitbrock, Institute for Immunology, Universtiy of Munich, Munich, Germany. The cell line can be obtained for research purposes only from Prof. Ziegler-Heitbrock or from the German Collection of Microorganisms and Cell Cultures (DSMZ) Braunschweig, Germany. The conditions for licensing of the cell line are to be negotiated individually with Prof.Ziegler-Heitbrock.

* *Section 12. There were a number of discrepancies among the cited articles, the bibliography, and the provided references.*

This was resolved. Cited articles are listed in Section 12, articles in bold are attached as hardcopies in newly created Appendix B. Appendix B includes the same set of publications for the 5 BRDs.

* *The Section 12 reference list contains 27 references; 15 of which are in Section 15. There are 13 articles included in Section 15 that are not listed in Section 12; one of these is also on the CD file. There are 6 articles on the CD file, one of which is also listed in Section 12, and another of which is included as hard copy in Section 15.*

see above