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Research paper

International validation of novel pyrogen tests based on human monocytoid cells

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Abstract

It is a requirement that parenteral medicines be tested for pyrogens (fever causing agents) using one of two animal-based tests: the rabbit pyrogen test and the bacterial endotoxin test. Understanding the human fever reaction has led to novel non-

Abbreviations: BET, bacterial endotoxins test; CI, confidence interval; DL, developing laboratory; ECVAM, European Centre for the Validation of Alternative Methods; ELC, endotoxins limit concentration; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; IFNγ, interferon γ; IL, interleukin; LAL, limulus amoebocyte lysate; LPS, lipopolysaccharide; LTA, lipoteichoic acid; LoD, limit of detection; MM6, MONO MAC 6; MVD, maximum valid dilution; NL, naive laboratory; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; TLR, toll-like receptor; TNFα, tumor necrosis factor α; WBT, whole blood test.

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animal alternative tests based on in vitro activation of human monocytoid cells in response to pyrogens. Using 13 prototypic drugs, clean or contaminated with pyrogens, we have validated blindly six novel pyrogen tests in ten laboratories. Compared with the rabbit test, the new tests have a lower limit of detection and are more accurate as well as cost and time efficient. In contrast to the bacterial endotoxin test, all tests are able to detect Gram-positive pyrogens. The validation process showed that at least four of the tests meet quality criteria for pyrogen detection. These validated in vitro pyrogen tests overcome several shortcomings of animal-based pyrogen tests. Our data suggest that animal testing could be completely replaced by these evidence-based pyrogen tests and highlight their potential to further improve drug safety.

Keywords: Pyrogens; Validation study; Cytokines; Monocytes; Alternatives to animals; Cell culture

1. Introduction

Pyrogens, a chemically heterogeneous group of fever-inducing compounds, are derived from bacteria, viruses, fungi or even from the host. Monocytes/macrophages react to microbial products during an immune response by producing endogenous pyrogens such as prostaglandins and the proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) (Dinarello, 1999). Depending on the type and amount of pyrogen challenge and the sensitivity of an individual, life-threatening shock-like conditions can be provoked. Consequently, to assure the quality and safety of any pharmaceutical product for parenteral application in humans, pyrogen testing is mandatory.

Depending on the drug, one of two animal-based pyrogen tests is currently prescribed by the health authorities and Pharmacopoeias, i.e., the rabbit pyrogen test or the bacterial endotoxins test (BET), often referred to as the Limulus amebocyte lysate test (LAL). For the rabbit pyrogen test, sterile test substances are injected intravenously into rabbits and any rise in body temperature is measured. This in vivo test detects various pyrogens but the fact that large numbers of animals are required to identify the rare pyrogen-containing samples in routine practice argues against its use if valid alternatives are available. In the past two decades, the declared intention to refine, reduce and replace animal testing, the 3Rs concept (Russel and Burch, 1959) that was implemented e.g. into European legislation in 1986 (European Union, 1986), has led to a reduction in rabbit pyrogen testing by 80% by allowing the BET as an in vitro alternative pyrogen test for many parenteral products.

Bacterial endotoxin consists largely of lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria and stimulates monocytes/macrophages via interaction with CD14 and toll-like receptor 4 (TLR4) (Beutler and Rietschel, 2003). It is the pyrogen of major concern to the pharmaceutical industry due to its ubiquitous sources, its stability and its high pyrogenicity (Mascoli and Weary, 1979a,b; Twohy et al., 1984). With the BET, endotoxin is detected by its capacity to coagulate the amoebocyte lysate from the haemolymph of the American horseshoe crab, *Limulus polyphemus*, or the Japanese horseshoe crab, *Tachypleus tridentatus*, a principle recognized some 40 years ago (Levin and Bang, 1964). In the United States, *Limulus* crabs are generally released back into the wild after drawing about 20% of their blood and therefore most of these animals survive. However, the procedure still causes mortality of about 30,000 horseshoe crabs per year, which adds to the even more severe threats to the horseshoe crab population such as their use as bait for fisheries, habitat loss and pollution (http://www.horseshoecrab.org). As with the rabbit test the general problem of translation of the test results to the human fever reaction persists. Moreover, although it is highly sensitive, the failure of the BET to detect non-endotoxin pyrogens as well as its susceptibility to interference by, for example, high protein levels of test substances or by glucans impedes full replacement of the rabbit pyrogen test (Roslansky and Novitsky, 1991; Fennrich et al., 1999). Hence, an estimated 200,000 rabbits per year are still used for pyrogen testing in the European Union.

A test system that combines the high sensitivity and in vitro performance of the BET with the wide

range of pyrogens detectable by the rabbit pyrogen test is therefore required in order to close the current testing gap for pyrogens and to avoid animal-based tests. With this intention and due to improved understanding of the human fever reaction (Dinarello, 1999), test systems based on in vitro activation of human monocytoid cells have been developed. First efforts date back about 20 years, when peripheral blood mononuclear cells (PBMC) were used to detect endotoxin by monitoring the release of pyrogenic cytokines (Duff and Atkins, 1982; Dinarello et al., 1984). Subsequently, a number of different test systems, using either whole blood, PBMC or the monocytoid cell lines MONO MAC 6 (MM6) (Ziegler-Heitbrock et al., 1988) or THP-1 (Tsuchiya et al., 1980) as a source for human monocytes and various read-outs have been established and were recently reviewed (Poole et al., 2003). Here, the six most prominent of these test systems were formally validated with the aim of developing an evidence-based tool for safer, animal-free and more efficient pyrogen detection suitable for regulatory acceptance. Formal validation of in vitro methods, i.e. the evaluation of reliability and relevance of a method, was developed by the European Centre for the Validation of Advanced and Alternative Methods (ECVAM) and is now internationally accepted (Balls et al., 1990, 1995; Worth and Balls, 2002).

2. Methods

2.1. Rabbit pyrogen test

For this study data from 171 rabbits (kindly provided by Dr. U. Lüderitz-Püchel) accumulated over several years at the Paul Ehrlich Institute, the German Federal Agency for Sera and Vaccines in Langen, were used for analysis. For these experiments, Chinchilla Bastards (Charles River) were injected with 0, 5, 10, 15 and 20 endotoxin unit (EU) in 1 ml/kg of *Escherichia coli* LPS (EC5 (Poole and Mussett, 1989) or EC6 (Poole et al., 1997)) in saline (corresponding to 0, 0.5, 1.0, 1.5 and 2.0 EU/kg in 10 ml, the largest volume allowed for injection in rabbits). The fever threshold in rabbits was defined as a body temperature increase of 0.55 °C during 180 min after injection. This value represents the mean individual rabbit value at the threshold of 6.6 °C of the EP when the maximum of twelve animals is tested (Council of Europe, 2001b).

2.2. In vitro monocyte-based tests

Good laboratory practice and concordant Standard Operating Procedures of the various methods were made available by ECVAM (http://ecvam.jrc.it). The test systems have been summarized by Hartung et al. (2001) and detailed in previous work (Poole et al., 1988; Taktak et al., 1991; Eperon and Jungi, 1996; Hartung and Wendel, 1996; Peterbauer et al., 1999; Peterbauer et al., 2000).

2.3. Reagents and consumables for all methods

The 2nd International WHO Standard for endotoxin (from *E. coli* O113:H10:K(-) (94/580), which is identical to FDA/USP standard EC6/Lot G was used as the standard endotoxin (Poole et al., 1997). Test materials for validation are specified in the Results section. All consumables were purchased as sterile and pyrogen-free and non specified reagents were pro analysis grade.

2.4. PBMC–IL6

2.4.1. Blood collection and preparation of PBMC

Blood donors had to describe themselves as being in good health, not suffering from any bacterial or viral infections for at least one week prior to the donation of blood and not taking drugs known to influence the production of cytokines. Using a heparinized (50 μl Fragmin at 10,000 IU, Dalteparin, Pharmacia) syringe, 30 ml blood were collected. Within 2 h, PBMCs were isolated from 20 ml Lymphoprep (Nycomed, Oslo, Norway), 15 ml PBS and 15 ml of heparinized whole blood by centrifuging at 340 ×g for 45 min at room temperature. The PBMC-layer was washed twice with PBS, centrifuging at 340 ×g for 15 min. The sediment was suspended with RPMI-C (RPMI 1640, Life Technologies™, Paisley, Scotland) with 10 ml/l human serum AB from clotted human male whole blood (Sigma), 10 ml/l L-Glutamine (Life Technologies™), 200 mM, and 20 ml/l Penicillin/Streptomycin solution

(Seromed, Vienna, Austria)) after counting in a Neubauer haemocytometer to 1 mio cells/ml. The cells were incubated with samples within 4 h of blood withdrawal.

2.4.2. Protocol for PBMC–IL6

For each of four blood donors, 100 μl of RPMI-C, 50 μl of samples/controls and 100 μl of gently swirled PBMC were incubated in quadruplicate in a 96-well tissue culture plate (Falcon Microtest, Becton Dickinson Labware) at 37 °C for 16–24 h in an atmosphere of 5% CO_2 in humidified air. After incubation, 50 μl of supernatant from each of the wells were transferred to the ELISA plate ensuring that cells were not aspirated by angling the assay plate.

2.4.3. ELISA for PBMC–IL6

A 2.5 μg/ml coating mouse monoclonal anti-IL-6 antibody (Novartis in-house Clone 16) was added at 200 μl to each well of a 96-well microtiter plate (Nunc-Immuno 96-well plate MaxiSorp, F96; Life Technologies™) at 15–25 °C for 16–24 h. The washed plate was coated with 200 μl blocking buffer (24.2 g/l Tris(hydroxymethyl)aminomethane, 0.2 ml/l Kathon MW/WT (Christ Chemie AG, Reinach, Switzerland) and 10.0 g/l bovine serum albumin). Plates were incubated with 200 μg/ml horseradish peroxidase conjugated to sheep anti-IL-6 antibodies (Novartis, in-house) for 2–3 h at 20–25 °C. Shortly before use, 90 ml substrate buffer and 4.5 ml TMB solution (240 mg 3,3′,5,5′Tetramethylbenzidine in 5 ml acetone, 45 ml ethanol and 0.3 ml Perhydrol (30% H_2O_2)) were mixed and 200 μl pipetted into each well. After 10–15 min, the enzyme reaction was stopped by 50 μl of 5.4% H_2SO_4 per well. The absorbance values were measured at 450 nm using 540–590 nm as reference wavelength.

2.5. WBT–IL1

2.5.1. Blood collection for WBT–IL1

Blood donors were required to show no evidence of disease or need for medication during the last two weeks. Blood was collected into heparinized tubes (Sarstedt S-MONOVETTE 7.5 ml, 15 IU/ml Li–Heparin) and used within 4 h (Schins et al., 1996).

2.5.2. Protocol for WBT–IL1

In the following order and in quadruplicate per single blood donor, 1000 μl saline, 100 μl sample/control and 100 μl blood were added to pyrogen-free reaction tubes (Greiner Bio-one tubes, 1.2 ml (polystyrene) or 1.5 ml (polypropylene), Frickenhausen, Germany). Closed tubes were mixed gently, inverted once or twice and then incubated in an incubator or a heating block at 37±1 °C for 10–24 h. The incubation tubes were mixed thoroughly by inverting them. Incubations were centrifuged for 2 min at 10,000 ×g and the clear supernatant (in aliquots of ≥150 μl) was used for the ELISA (ENDOSAFE-IPT, Charles-River Endosafe, Charleston, USA) following the manufacturer's procedure.

2.6. WBT–IL6

2.6.1. Blood collection for WBT–IL6

Blood donors were selected as described for PBMC–IL6. Thirty milliliters of blood were drawn and immediately transferred into a 50 ml sterile centrifuge tube containing 300 IU heparin (Fragmin, Pharmacia, diluted 1/10 with saline). The closed tubes were inverted slowly five times to ensure thorough mixing without vortexing and used within 4 h (Schins et al., 1996).

2.6.2. Protocol for WBT–IL6

For each of four blood donors, 50 μl of saline, 50 μl of gently mixed blood, 50 μl of samples/controls and 100 μl of saline were incubated in quadruplicate in a 96-well tissue culture plate (Falcon Microtest, Becton Dickinson Labware) at 37 °C for 16–24 h in a humid atmosphere of 5% CO_2. After incubation, 50 μl of supernatant from each of the wells were transferred to the ELISA plate ensuring that cells were not aspirated by angling the assay plate. The same IL-6 ELISA as for PBMC–IL6 was used.

2.7. MM6–IL6

2.7.1. Cell culture for MM6–IL6

The human monocytoid cell line MonoMac-6 was obtained from Prof. H.W.L. Ziegler-Heitbrock (Institute for Immunology, University of Munich, Munich,

Germany). Frozen cells from liquid nitrogen were thawed on ice. Cells were transferred to a 50 ml centrifuge tube, 10 ml RPMI (+4 °C) (Life Technologies™) were added and then centrifuged at 100 ×g for 5 min at +4 °C. Afterwards the cells were resuspended in 10 ml RPMI-M (containing 10% ml heat-inactivated low-pyrogen foetal calf serum, 2 mM L-Glutamine, 0.1 mM MEM nonessential amino acid, 0.23 IU/ml Bovine insulin, 1 mM Oxaloacetic acid, 1 mM Sodium pyruvate, 20 mM HEPES). After a wash step, cells were transferred to a 25 cm^2 tissue culture flask and incubated at 37 °C, with 5% CO_2 and high humidity. The number of viable cells was determined by Trypan blue exclusion using a haematocytometer. The cells were passaged with 2×10^5 cells/ml twice a week.

2.7.2. Protocol for MM6–IL6

To pre-incubate the cells for a test, 30–50 ml of cell suspension were centrifuged at 100 ×g for 8 min at room temperature and resuspended in RPMI-C (as RPMI-M, but only 2% heat-inactivated foetal calf serum) at a final concentration of 4×10^5 cells/ml. The cells were incubated for approximately 24 h at 37 °C, 5% CO_2 and high humidity. Cells were washed and counted as above, diluting to 2.5×10^6 viable cells/ml, just prior to addition to the culture plate. In quadruplicate tests, 50 μl of samples/controls, 100 μl of RPMI-C and 100 μl of gently swirled MM6 were incubated in 96-well tissue culture plates at 37 °C for 16–24 h with 5% CO_2 and humidified air. After incubation, 50 μl of supernatant from each of the wells were transferred to the ELISA plate ensuring that cells were not aspirated by angling the assay plate. The same IL-6 ELISA as for PBMC–IL6 was used.

2.8. THP–Neo

2.8.1. Cell culture for THP–Neo

THP-1 cells were obtained from the American Type Culture Collection (ATCC, TIB-202). The 6×10^6 cells were seeded in 60 ml medium (RPMI 1640 supplemented with 10% (v/v) FCS (high-quality lots with the lowest endotoxin content available (<30 pg/ml) were chosen, e.g. Biochrom, Berlin, Germany) in 75 cm^2 culture flasks. Flasks were incubated in the upright position at 37 °C with 5% CO_2 and humidified air. On the fourth day of culture, a further 30–60 ml (depending on the culture doubling time) of culture medium were added and cells were incubated for another three days. If cells from freshly thawed stocks are used, they were grown for two to three weeks in order to ensure that they divided properly before using them for tests. Furthermore, cells were not kept in culture for more than four months but new cultures were started from frozen stocks at regular intervals. Cells were counted with a hemocytometer and cell viability by trypan blue exclusion was ≥90%. Tubes with 2.5×10^7 cells (for one plate) were centrifuged at 400 ×g and 20 °C for 7 min and resuspended in 20 ml medium, 2 mM L-glutamine and 50 μM 2-mercaptoethanol.

2.8.2. Protocol for THP–Neo

One hundred microliters of IFNγ (human, recombinant, endotoxin content <0.1 EU/mg; Gammaferon 50, Rentschler Biotechnologie, Laupheim, Germany) stock solution (6250 U in 100 μl medium, 110 μl aliquots) were added to 20 ml of cell suspension and mixed well. Two hundred microliters/well of mixed cell suspension were added to a 96-well cell culture microtiter plate. After incubation for 30 min, 50 μl of vortexed samples/controls (in quadruplicate) were added and put on an orbital plate shaker for 2 min at room temperature and 500 rpm. After 18–22 h of incubation, 150 μl of supernatant were collected and frozen and/or directly processed with the neopterin ELISA (Elitest Screening, Brahms Diagnostica, Berlin, Germany) according to the manufacturer's protocol.

2.9. THP–TNF

2.9.1. Protocol for THP–TNF

THP-1 cells (obtained from ATCC or ECACC) were used. Subclones from this cell line prepared in-house showed a higher sensitivity towards LPS. Cells were cultured in RPMI (1% L-glutamine, 1% HEPES, 1% Penicillin/streptomycin solution, 1% Sodium pyruvate, all from Biochrom (Berlin, Germany), 1% nonessential amino acids for MEM, 0.4% MEM vitamin solution, 0.5% β-mercaproethanol (10 mM), all from Invitrogen (Basle, Switzerland), and 12% heat-inactivated low-pyrogen FCS in 6-well plates or

T25 flasks at 37 °C in a humidified 5% CO_2 incubator. They were passaged once weekly. When new cells were required for an assay, cells from a cryovial were thawed two to three weeks before use. For the last passage prior to the test, terminal differentiation was induced by culturing the cells in the presence of sterile-filtered calcitriol (1,25-dihydroxy vitamin D3, Sigma or Hoffmann-La Roche, Basle, Switzerland) (10 μg/ml) for 44–48 h. Cells were collected, centrifuged and resuspended in culture medium containing calcitriol (final concentration 100 ng/ml). They were counted and adjusted to $1–1.25\times10^6$ cells/ml. Cells were cultured for 44–48 h in T25 flasks. Then, terminally differentiated cells were harvested and counted using a haematocytometer and trypan blue. Cells were diluted to 1.25×10^6 cells/ml and 200 μl of suspension were dispensed into each well of the 96-well cell culture plate, described below, already containing 50 μl of sample/control (in quadruplicate). Plates were incubated for 16–24 h at 37 °C in an atmosphere of 5% CO_2.

2.9.2. TNFα ELISA for THP–TNF

Non-sterile plates Dynex PF microtiter 'flat bottom' styrene 96-well plates (Dynex Tech., Worthing, UK) were rinsed extensively with pyrogen-free PBS. The plates were coated with 1 μg/ml monoclonal antibody 101-4 against human TNFα (a generous gift from Dr. T Meager, Division of Immunobiology, NIBSC, UK) at 100 μl/well and 4 °C overnight. Fifty microliters of sample/control (in quadruplicate) or duplicate of TNFα standards (250, 62.5, 15.6, 3.9, 0.98, 0.24, 0 U/ml, NIBSC, UK) were added for 16–24 h at 37 °C in an atmosphere of 5% CO_2. An aliquot of the detecting antibody (biotinylated goat-anti-human TNFα from the Duoset kit, R and D, Minneapolis, USA) was diluted 180-fold, using dilution buffer (0.1% bovine serum albumin, 0.1% Tween 20, in 20 mM Tris, 100 mM NaCl, pH 7.2–7.4). One hundred microliters were dispensed into each well for 2 h at room temperature. After washing, 100 μl Streptavidin-peroxidase conjugate (R and D, Minneapolis, USA) were added for 20 min. After washing, 100 μl of TMB (Sigma) were dispensed and incubated in the dark before reading absorbance values at 650 nm. Incubation times were chosen so that 250 U/ml TNFα had an absorbance of >1.5.

2.10. Data analysis

The rabbit fever reaction was modeled by regression techniques applied to the logarithmically transformed data. The within- and between-laboratory reproducibility were assessed comparing the resulting classifications by means of simple matching, i.e. the proportions of identically classified samples, as a measure of similarity. For the within-laboratory reproducibility, where three independent but identical runs were performed, the mean similarity was calculated.

A one-sided *t*-test, designed to establish the safety of a tested compound, was employed as a so-called prediction model (PM) to dichotomize the test results into a classification of either 'pyrogenic' or 'non-pyrogenic'. The *t*-test compared the data of a given sample against the data of the standard positive control of 0.5 EU/ml, which was performed in parallel. It was calculated with the log-transformed data and a local significance level of 1% was chosen in order to increase safety. If this test resulted in a significant *p*-value, i.e. smaller than 1%, then the considered sample was classified as non-pyrogenic, and as pyrogenic if otherwise. This means that a negative sample had to be significantly lower than 0.5 EU/ml. The levels of contamination chosen were 0, 0.25, 0.5 (twice) and 1 EU/ml. According to the rabbit model, 0 and 0.25 EU/ml were considered as non-pyrogenic samples and 0.5 and 1 EU/ml as pyrogenic samples. Having thus defined the reference standard, i.e. the 'true' contamination level, we calculated via 2×2-contingency tables the performance parameters sensitivity, i.e. the probability of a correct positive classification, and specificity, i.e. the probability of a correct negative classification. Confidence intervals for these parameters were calculated with the Clopper and Pearson method based on the *F* distribution (Clopper and Pearson, 1934).

3. Results

3.1. The limit of endotoxin detection in rabbits

Employing regression techniques, the temperature data from 171 rabbits could be modeled by the equation $y=0.217*(EU+1)^{0.508}$, where y is the

expected temperature increase for a given concentration in EU/ml (Fig. 1). This approach was recently described in more detail and further exploited (Hoffmann et al., 2005, in press). The model indicated that 50% of the animals developed fever, i.e. showing a 0.55 °C rise of body temperature within 180 min after injection, in response to 5.22 EU per kg body weight of endotoxin with a 95%-confidence interval ranging from 4.24 to 6.21 EU/ml. Only at 20 EU per kg of body weight, did all animals show an increase in temperature of 0.55 °C or more. We deduced from these data that a sample concentration of 0.5 EU/ml represents the required limit of detection (LoD) that alternative pyrogen tests must meet. This assumption takes into account the fact that the largest volume allowed for injection into rabbits is 10 ml/kg, corresponding to 0.5 EU/ml for injections at 10 ml/kg. Thus, the concentration of 0.5 EU/ml was defined as the threshold between pyrogenic and non-pyrogenic samples.

3.2. Prevalidation of the novel in vitro pyrogen tests

Before prevalidation, the test-developing laboratories that took part in the study compiled standard operating procedures for the alternative tests. This required an intensive phase of test optimization and standardization in order to allow the transfer of the tests. A standard curve of endotoxin in saline, including the 0.5 EU/ml concentration as the threshold for pyrogenicity, was included in all tests. Only if the 0.5 EU/ml endotoxin standard was detectable, did the test run qualify for analysis. Before prevalidation was started, the naive laboratories provided evidence of successful transfer of the respective test systems (data not shown). Prevalidation was then carried out with twelve blinded samples. These consisted of three drugs spiked with either pyrogen-free saline (clinical grade 0.9% NaCl) or with reference endotoxin. Two negative, i.e. pyrogen-free samples, and two LPS-containing, i.e. pyrogenic samples (0.5 EU/ml and 1.0 EU/ml sample concentration, respectively) were tested. The concentration of 0.5 EU/ml was the limit of detection defined for the rabbit pyrogen test (see above). The drugs used were Gelafundin®, a volume-replacement therapy for transfusion with high protein (gelatine) content (B. Braun Melsungen AG, Melsungen, Germany), Jonosteril®, an electrolyte infusion (Fresenius AG, Bad Homburg, Germany) and Haemate®, a factor VIII preparation (Aventis Behring GmbH, Marburg, Germany). In addition, a positive control (0.5 EU/ml LPS in saline) and a negative control (endotoxin-free saline) were included. Each test was performed three times in the respective developing laboratory (DL) as well as in two naive laboratories (NL).

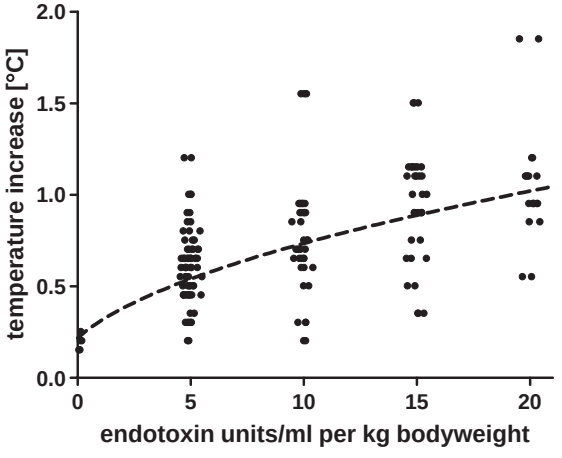

Fig. 1. Temperature increase of 171 rabbits upon endotoxin injection with a fitted regression line. The maximum temperature increase in °C within 180 min after endotoxin injection of 171 rabbits is presented. The mean temperature increase, modeled with regression techniques, is indicated by the dotted line.

Table 1 summarizes the six novel test systems used, their major characteristics, their performance regarding reproducibility, which was assessed before the blinding code was broken, as well as sensitivity and specificity. As can be seen, the predictive capabilities of the various tests were encouraging, particularly in the light of the restricted stability of endotoxin spikes at the borderline concentration of 0.5 EU/ml. Although all tests were successfully transferred to the naive laboratories during the preparatory phase of prevalidation, this optimal performance could not be maintained for the two test systems using THP-1 cells, as is reflected by the comparatively low between-laboratory reproducibility between the developing laboratory and one of the naive laboratories for each. The lower specificity of the THP–Neo test was entirely caused by misclassification in NL2. Furthermore, prevalidation also revealed that, despite preceding interference testing and diluting of the drugs accordingly, interference/recovery problems

Table 1
Novel pyrogen tests and their performance in prevalidation

Test	System	Read-out	Ref.	Within-laboratory reproducibility (%)	Between-laboratory reproducibility (%)	Sensitivity (%)	Specificity (%)
WBT–IL6	Whole blood	IL-6	(Poole et al., 2003)	DL: 83.3 NL1: 94.4 NL2: 100	DL–NL1: 72.2 DL–NL2: 72.2 NL1–NL2: 96.3	72.2	92.6
WBT–IL1	Whole blood	IL-1β	(Taktak et al., 1991)	DL: 88.9 NL1: 95.8 NL2: 94.4	DL–NL1: 91.7 DL–NL2: 76.8 NL1–NL2: 67.8	72.0	100.0
PBMC–IL6	PBMC	IL-6	(Hartung and Wendel, 1996)	DL: 94.4 NL1: 100 NL2: 94.4	DL–NL1: 80.6 DL–NL2: 86.1 NL1–NL2: 88.9	87.0	98.1
MM6–IL6	MM6 (Ziegler-Heitbrock et al., 1988)	IL-6	(Poole et al., 2003)	DL: 100 NL1: 94.4 NL2: 94.4	DL–NL1: 97.2 DL–NL2: 88.9 NL1–NL2: 86.1	72.2	100.0
THP–TNF	THP-1 clone	TNFα	(Eperon and Jungi, 1996)	DL: 94.4 NL1: 83.3 NL2: 55.5	DL–NL1: 90.7 DL–NL2: 67.6 NL1–NL2: 65.7	66.7	88.9
THP–Neo	THP-1 parental (Tsuchiya et al., 1980)	Neopterin	(Eperon and Jungi, 1996)	DL: 100 NL1: 94.4 NL2: 77.7	DL–NL1: 97.2 DL–NL2: 50.0 NL1–NL2: 51.8	88.9	72.2

Protocols for all methods are listed in Poole et al. (2003) and in the Methods section. All tests include dilution of the sample by 1/5 with the exception of the WBT–IL1 test that requires a 1/12 dilution of the sample. The WBT–IL6 and the PBMC–IL6 tests combine data from three and four blood-donors per run, respectively; the WBT–IL1 test data were from one donor per run. Samples and controls were tested in quadruplicate in each of the tests. DL denotes developing laboratory, NL1 and NL2 the two naive laboratories. The sample size analyzed for sensitivity and specificity was 108 for all tests apart from WBT–IL1 (100 samples). Sensitivity describes the probability of correctly classifying positive samples and specificity describes the probability of correctly classifying negative samples.

persisted in some cases, as is reflected by the values for sensitivity.

3.3. Validation phase

For the validation phase, 10 drugs with five blinded spikes each (0 (i.e. pyrogen-free), 0.25, 0.5 (twice) and 1 EU/ml) were tested, again in three laboratories, i.e. the DL of a test and the two NLs, respectively. To avoid the possibility that different dilutions of the drugs were tested depending on their different interference with different test systems, all drugs were tested at their maximum valid dilution (MVD), thus adopting the rationale of the pharmacopoeial BET reference (limit) test. The MVD is calculated from the endotoxin limit concentration (ELC in EU/ml) defined for a drug by the European Pharmacopoeia (Council of Europe, 2001a), divided by the threshold of pyrogenicity as the limit of detection (LoD), i.e. 0.5 EU/ml. Drugs, sources, ELCs and MVDs (=ELCs/LoD, where LoD=0.5) are summarized in Table 2.

While the tests using whole blood, PBMC and MM6 cells performed well in all three test laboratories in terms of reproducibility (Table 3), technical problems with the two tests using THP-1 cells were obvious. For the THP–TNF test this was caused by a batch of TNFα-ELISA plates sent out to the two NLs that did not satisfy the quality criteria with regard to detection limit when used with cells. For the THP–Neo test, the technical problems in NL2 persisted such that the quality criteria defined in the SOP were not met. The tests could not be repeated due to the limited time frame of validation and for logistical reasons. Therefore, for the THP–TNF assay only the data from the DL and for the THP–Neo assay only the data from the DL and from NL1 could be analyzed. Sensitivity and specificity were 76.7% and 78.9% for the THP–TNF assay (sample size=40) and 93.3% and 47.5% for the THP–Neo assay (sample size=100). The data for the other four tests are summarized in Table 3. Almost all misclassifications, either false negatives or false positives, occurred around or at the defined classi-

Table 2
Test substances for the validation phase

Drug	Source	Agent	Indication	ELC (EU/ml)	MVD (-fold)
Glucose 5% (w/v)	Eifelfango GmbH	Glucose	Nutrition	35	70
Ethanol 13% (w/v)	B. Braun AG	Ethanol	Diluent	17.5	35
MCP®	Hexal AG	Metoclopramid	Antiemetic	175	350
Orasthin®	Aventis Pharma GmbH	Oxytocin	Initiation of delivery	350	700
Binotal®	Aventis Pharma GmbH	Ampicillin	Antibiotic	70	140
Fenistil®	Novartis Consumer Health GmbH	Dimetindenmaleat	Antiallergic	87.5	175
Sostril®	GlaxoSmithKline GmbH	Ranitidine	Antiacidic	70	140
Beloc®	Astra Zeneca GmbH	Metoprolol tartrate	Heart dysfunction	70	140
Drug A		0.9% NaCl		17.5	35
Drug B		0.9% NaCl		35	70

Drugs were obtained from Eifelfango GmbH (Bad Neuenahr-Ahrweiler, Germany), B. Braun AG (Melsungen, Germany), Hexal AG (Holzkirchen, Germany), Aventis GmbH (Bad Soden, Germany), Novartis GmbH (München, Germany), GlaxoSmithKline GmbH (München, Germany) and Astra Zeneca GmbH (Wedel, Germany). ELCs of drugs were calculated according to European Pharmacopoeia (Council of Europe, 2001a). Drugs were selected by a selection committee which excluded the developing laboratories and included experts. Drugs A and B which were saline only were included as further controls using notional ELCs.

fication threshold, i.e. for the contaminations of 0.25 and 0.5 EU/ml. Confidence intervals (CI) with a significance level of 5% were calculated for sensitivity and specificity. By focusing on the lower bounds of CI (Fig. 2), a worst-case scenario can be considered by which the likelihood of underestimation of pyrogen content is maximized and thus possible negative consequences for health can be estimated. The lower predictive capability of the WBT–IL1 test as compared to the WBT–IL6 and the PBMC–IL6 test can be explained by the one-donor approach used for the WBT–IL1 test. The multiple-donor approach used for the other tests is more conservative and laborious, but decreases the probability of false-negative classifications. For the THP–TNF assay, the lower bounds of CI for sensitivity and specificity were 60.6% and 55.2%, respectively. For the THP–Neo assay, the respective lower bounds were 78.0% and 38.7%. Applying this kind of analysis to the in vivo assay employing the regression model based on the data from the rabbit pyrogen test yields a sensitivity of 57.8% and a specificity of 88.3% (Table 3) with confidence intervals also presented in Fig. 2. Thus, the novel

Table 3
Validation of the predictive capability of novel pyrogen tests

Test	Between-laboratory reproducibility (%)	Sample size: sensitivity[a]	Sensitivity (%)	Sample size: specificity	Specificity (%)
WBT–IL6	DL–NL1: 85.4 DL–NL2: 85.4 NL1–NL2: 92.0	89	88.9	59	96.6
WBT–IL1	DL–NL1: 72.9 DL–NL2: 81.6 NL1–NL2: 70.2	88	72.7	59	93.2
PBMC–IL6	DL–NL1: 84.0 DL–NL2: 86.0 NL1–NL2: 90.0	90	92.2	60	95.0
MM6–IL6	DL–NL1: 90.0 DL–NL2: 89.6 NL1–NL2: 83.3	89	95.5	59	89.8
Rabbit[b]	–	–	57.9	–	88.3

[a] Sample sizes are reduced by outlier exclusion defined in the study protocol.
[b] Parameters calculated by the fitted regression model.

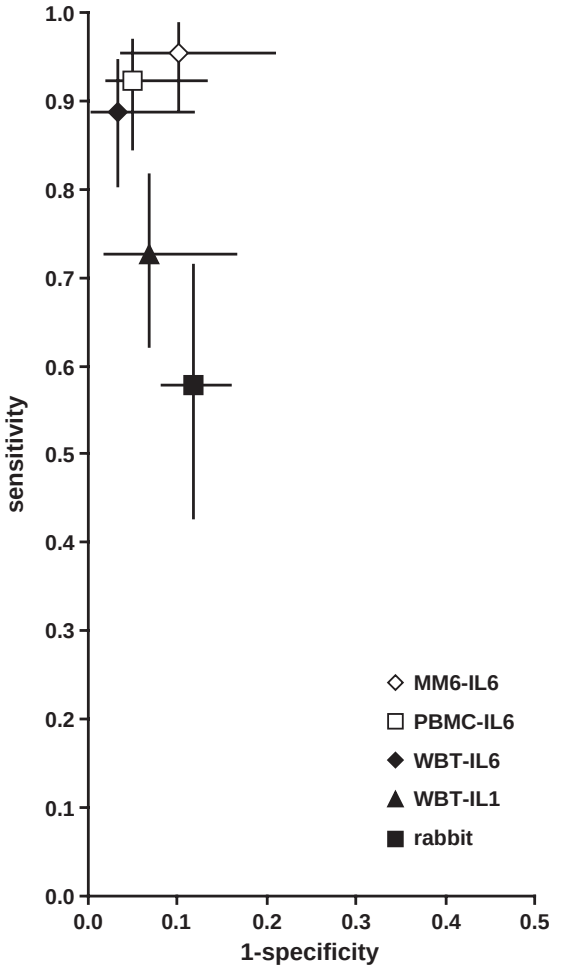

Fig. 2. Sensitivity and specificity of four in vitro assays in the validation study and modeled rabbit test performance with 95%-confidence intervals. The sensitivity and specificity resulting from the pre-defined prediction model and considering samples with 0 and 0.25 EU/ml as non-pyrogenic and with 0.5 and 1 EU/ml as pyrogenic are presented with their corresponding 95%-confidence intervals for four validated tests. Similarly, the respective parameters were calculated with the rabbit model. As performance improves towards the upper left of the graph, all validated tests outperform the rabbit test.

pyrogen tests listed in Table 3 show parameters of performance exceeding the rabbit pyrogen test.

An additional analysis, which could be conducted with the available data, supports this conclusion. According to their SOPs, the four systems included an uncontaminated negative control, i.e. saline, and another positive control of 1 EU/ml. For each of these two controls we adapted the prediction model described above. First, we compared the blinded samples against the response of the 1 EU/ml control. Therefore, we constructed a modified prediction model using the 1 EU/ml control response instead of the positive control of 0.5 EU/ml, which is denoted in the following by PM1. In doing so, the true classification of the samples changed, as now only the samples spiked with 1 EU/ml were considered as pyrogenic and the other samples as non-pyrogenic. Second, with a modified prediction model, denoted as PM0, classifying a sample as pyrogenic when the response was significantly larger than the negative control response (significance level 1%), we compared all spikes against this control. Again, the true classification of the samples needed to be adjusted considering the contaminated samples (0.25, 0.5, 1.0 EU/ml) as pyrogenic and the unspiked samples as non-pyrogenic. The resulting sensitivities and specificities are summarized together with the results from the original PM for the four test systems in Fig. 3. All tests performed best for PM0, where the sum of these two parameters was at least 1.90, while WBT-IL6 even resulted in the maximum sum of 2.

The DLs also tested lipoteichoic acid (LTA) from *Bacillus subtilis*, a BET-negative Gram-positive compound that activates cytokine release from human monocytes (Morath et al., 2001, 2002) prepared according to Morath et al. (2002), which was clearly detectable by the novel tests.

4. Discussion

Previous work (Taktak et al., 1991; Eperon and Jungi, 1996; Hartung et al., 2001; Hartung and Wendel, 1996; Nakagawa et al., 2002; Peterbauer et al., 1999; Poole et al., 1988, 2003) has established that different sources of human monocytoid cells are valuable tools to mimic the human fever reaction in vitro. Not only can these cells detect the important pyrogen LPS from *E. coli* and other Gram-negative bacteria but also a number of compounds involved in the immune response to Gram-positive bacteria such as LTA (Schindler et al., 2003), exotoxins (Eperon et al., 1997; Peterbauer et al., 1999), cell wall components such as muramyl dipeptide (Eperon et al., 1997) or peptidoglycan (Harada et al., 1968), *S.*

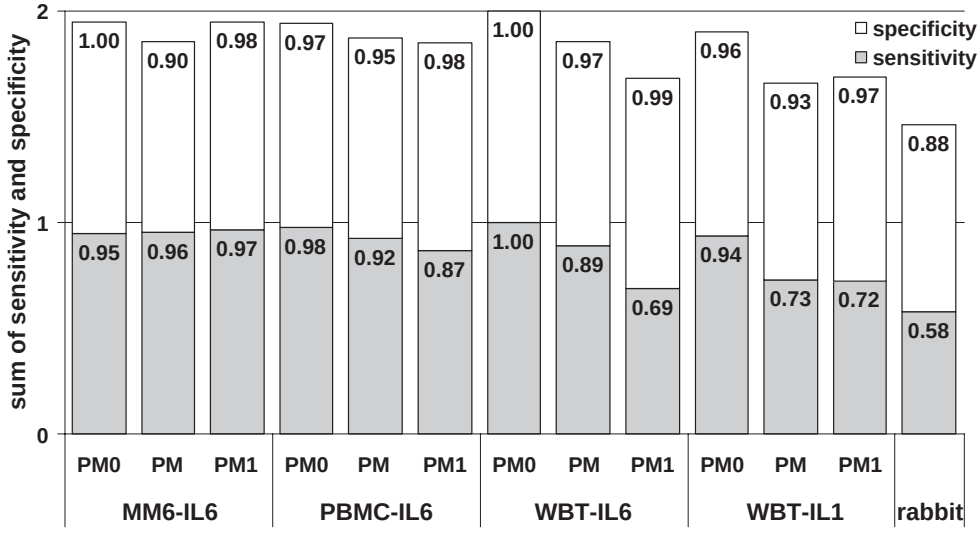

Fig. 3. Sum of sensitivity and specificity resulting from three prediction models for four in vitro assays in the validation study. The validation data of four tests were analyzed with three prediction models employing different controls for comparison and thus defining the true classification of the samples (non-pyrogenic vs. pyrogenic) accordingly. The test accuracy is described for each test and prediction model by the sum of both the specificity and sensitivity allowing also for individual parameter assessment. For comparison, the rabbit test performance according to the pre-defined prediction model is added.

aureus Cowan (SAC) (Nakagawa et al., 2002) or DNA (Peterbauer et al., 1999) as well as poly(I:C) (Nakagawa et al., 2002), a synthetic double-stranded RNA used as a virus model compound in fever research. It was also established that these novel test systems overcome limitations of the BET and yield results comparable to the rabbit pyrogen test (Eperon et al., 1997; Peterbauer et al., 1999; Nakagawa et al., 2002; Spreitzer et al., 2002). For the first time, six of these monocytoid-cell-based in vitro pyrogen tests were formally validated. For this purpose, a harmonized analysis procedure was established that allowed the direct comparison of the different tests and incorporated various safety aspects. A conservative statistical approach showed that four test systems met the criteria for safe detection of pyrogens. The two test systems based on the use of THP-1 cells posed problems in performance. These were related to insufficient transfer to one naive laboratory (THP–Neo) and to the use of an ELISA batch for the one-plate assay format (THP–TNF) that, although qualifying for the detection of TNFα did not qualify for their use with cells and caused prestimulation. Both problems became obvious only during validation and could not be overcome within the tight schedule of validation. Thus, for these two systems additional validation processes would be required. However, the data obtained for the other four test systems clearly suggest that these have reached a stage of development that makes them suitable for use in pyrogen testing as replacements for the rabbit pyrogen test.

For the purpose of this study, a threshold value of 0.5 EU/ml was chosen on the basis of historical data from rabbit tests carried out in a national control authority. This approach was conservative as only 50% of animals of the very sensitive strain used showed a febrile reaction at this concentration. Additionally, in order to be classified as negative, the samples had to be, according to the PM, significantly lower than 0.5 EU/ml. On the one hand, the enormous challenge to the models by placing two samples at the threshold of 0.5 EU/ml, which had to be classified positive, resulted in reduced sensitivities. On the other hand, including a sample with 0.25 EU/ml, which had to be identified as negative, was the reason for almost all false-positive classifications resulting in the reduced specificities without representing any safety concern. When tested against the negative control (PM0), i.e. when samples which are not significantly

different from the negative control were considered as pyrogen-free, the tests performed even better, i.e. with increased sensitivity. However, this approach increases consumers' safety at the cost of rejecting drugs, whose minor pyrogenic contamination would not induce adverse health effects in humans. At the same time this reflects the fact that the study design placed the main emphasis on the threshold of 0.5 EU/ml. Similarly, decreased sensitivity when defining 1 EU/ml as a threshold value shows that the tests were especially designed for the threshold of 0.5 EU/ml. Since the test performance when changing the threshold is still acceptable or even better, the robustness of the alternative tests is emphasized.

In summary, this study provides evidence of the validity of these novel tests and should facilitate their regulatory acceptance and lead to their introduction into Pharmacopoeias.

5. Conflict of interest

S. Poole is named as an inventor in Patent Number US 6,696,261 B2, Feb 24, 2004: 'Pyrogenicity test for use with automated immunoassay systems'.

T. Hartung and A. Wendel are named as inventors in Patent Number US 5,891,728, Apr 6, 1999: 'Test for determining pyrogenic effect of a material'.

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