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Optimization and novel applications of the in vitro pyrogen test (IPT) using human whole blood

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Abbreviations

AAMI	American Association of Medical Instrumentation
AWIPT	absorb and wash in vitro pyrogen test
BAL	bronchoalveolar lavage
BET	bacterial endotoxin test
cAMP	cyclic adenosinmonophospate
CD	cluster of differentiation
CFU	colony forming unit
COX	cyclooxygenase
CRP	C-reactive protein
CV	coefficient of variation
DIC	disseminated intravascular coagulation
DL	developing laboratory
DMSO	dimethyl sulfoxide
ECVAM	European Center for the Validation of Alternative Methods
ELC	endotoxin limit concentration
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin unit
GLP	good laboratory practice
HD	hemodialysis
HSA	human serum albumin
ICE	interleukin-1 converting enzyme
IL	interleukin
IPT	In Vitro Pyrogen Test
IU	International Unit
NIBSC	National Institute for Biological Standards and Controls
LAL	Limulus Amoebocyte Lysate
LBP	LPS-binding protein

LoD	Limit of Detection
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LVP	large volume parenteral
MID	minimum interference dilution
MM-6	Monomac-6
MVD	maximum valid dilution
PAMPs	pathogen-associated molecular patterns
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PEI	Paul Ehrlich Institute
PG	prostaglandin
POD	peroxidase
PPC	positive product control
NFKB	nuclear factor kappa B
NL	naive laboratory
NPC	negative product control
NSAID	non-steroidal anti-inflammatory drug
OD	optical density
OVLT	organum vasculosum laminae terminalis
PM	prediction model
PMN	polymorphonuclear
PTFE	polytetrafluorethylene
RT	room temperature
SOP	standard operating procedure
SVP	small volume parenteral
TLR	toll-like receptor
TMB	tetramethylbenzoate
TNF	Tumor Necrosis Factor
USP	United States Pharmacopoeia
WBT	whole blood test

WHO	World Health Organisation

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1 Introduction

1.1 Pyrogens

The term “pyrogen” derives from the greek word “pyros” (fire). Pyrogens are therefore substances that have been recognized to cause fever in the organism. The relation of bacteria and fever was first recognized by Semmelweis (1) and Lister (2). The association of fever and intravenous injection, on the other hand, dates back to the eighteenth century, when van Haller noticed that the injection of putrid materials caused severe fever reactions (3). Panum, with the help of Virchow, was the first to state that the substance responsible was heat-stable, water-soluble, alcohol-insoluble, and independent of the presence of living bacteria (4). The term “pyrogen” was apparently used first by Billbroth (5).

At the end of the 19th century, Centanni first reproducibly isolated a substance from a variety of Gram-negative bacteria which he called pyrotoxina, which was most probably the first purified endotoxin in history (6). Injection fevers associated with intravenously applied parenterals were first systematically investigated by Hort and Penfold in 1912 (7), who injected them intravenously into the rabbit, measured the fever reaction, and classified the bacteria into pyrogenic and non-pyrogenic. Basically, these were the first rabbit pyrogen tests. Seibert then proved that the fever reactions were caused by filterable, heat-stable pyrogens from Gram-negative bacteria, a finding which was later confirmed by Rademaker, who already stressed the importance of avoiding contaminations in parenterals and differentiated between the terms “sterile” and “pyrogen-free” (8, 9). World War II then brought the development of large volume parenterals as volume substitution for injured soldiers. The occurrence of severe fever reactions resulted in a collaborative study establishing the rabbit pyrogen test (10, 11) and its incorporation into the US Pharmcopoeia in 1942.

1.1.1. Lipopolysaccharide (endotoxin)

Endotoxin as a component of the cell walls of Gram-negative bacteria is the

most potent and the most extensively studied pyrogen. Due to the fact that Gram-negative bacteria are ubiquitous, contaminations of parenterals with endotoxin pose a constant threat to the health of patients. Endotoxins are released from the cell not only after lysis, but are shed constantly from the living bacterium as well (12). Lipopolysaccharide (LPS) is a highly purified (protein-free) form of endotoxin. Chemically, they are heat-stable substances with three distinct regions: the lipid A portion, which has been shown to be responsible for the pyrogenic activity (13, 14), the core polysaccharide, and the antigenic O-specific side chain. The biological activities of endotoxins do not restrict themselves to causing fever and other inflammatory reactions, but also include complement activation, hypotension, and activation of the coagulation system, all of which can lead to severe complications, up to hypovolemic shock, disseminated intravascular coagulation (DIC) and death.
A maximum endotoxin contamination of 50 pg/ml (0.5 ng/kg) was first published by the Bureau of Drugs in 1980 (15), apparently with no scientific study having been performed to confirm this very restrictive threshold. In 2005, a study at the Paul-Ehrlich Institute (PEI) in Germany fully confirmed this limit (16).

1.1.2. Non-endotoxin pyrogens

Substances that have pyrogenic properties but are not of an endotoxin nature include enterotoxins (17, 18), exotoxins, (19), viruses (20), peptidoglycan (21-23) and fungi (24, 25). Since Gram-positive bacteria are as frequent as Gram-negative bacteria, the pyrogens of the former can be a serious health hazard as well. A major component of the Gram-positive cell wall is the peptidoglycan, which consists of β-1,4 linked N-acetyl-D- glucosamin and N-acetyl muramic acid, and was shown to have pyrogenic properties similar to those of endotoxin (21). The other prominent pyrogen of Gram-positive bacteria called lipoteichoic acid (LTA) was successfully purified in an endotoxin-free and biologically active manner in 2001 (26).

1.2. Traditional pyrogen tests

1.2.1. Rabbit pyrogen test

The rabbit pyrogen test has been the gold standard in pyrogen testing since 1942, when it was introduced into the USP (United States Pharmacopoeia). The rabbit species was chosen by Seibert, who also discovered the pyrogenic principle (8). In 1941, the need for pyrogen testing of LVP (large volume parenterals) due to World War II caused the Committee of Revision of the USP to authorize the first USP collaborative study of pyrogens with pyrogen filtrates of *Pseudomonas aeruginosa*. The results of this study led to the incorporation of the rabbit test in the 12th edition of the USP in 1942. In its simplest form, the test involves measuring a rise in body temperature for 3 hours following intravenous injection of a test solution into the marginal ear vein at a volume of not more than 10 ml/kg. Temperature is to be measured by a clinical thermometer inserted into the rectum of the rabbit at a depth of not less than 7.5 cm. Rabbit breeds used for testing are New Zealand Whites, Belgium Whites, Chinchillas and Dutch Belts. Differences in sensitivities of various strains have been investigated by van Dijck et al (27). Animals of one single sex are preferred, and there have been reports about male rabbits being more sensitive to pyrogens than females (28).

The test is positive if the sum of the rises in three rabbits exceeds 2.65 °C. The rabbit has a labile thermoregulation and tends to give false-positive results. Also, the very rigid fixation and the handling (injection procedure) can cause a hyperthermia due to excitement. On the other hand, it has been reported that the fixation and lack of movement can cause a hypothermia yielding false-negative results (29). Comparisons between the reactivity of humans and rabbits in vivo by Greisman 1969 showed that the threshold towards three endotoxin preparations was comparable, but that the humans respond more vigorously than the rabbits (30).

1.2.1. Limulus amoebocyte lysate test (LAL)

When in contact with the lipid A portion of endotoxin, the amoebocytes from Limulus polyphemus (horseshoe crab) coagulate due to an enzymatic reaction (31, 32). In the presence of calcium, the clotting enzyme zymogen is activated by a serin protease and acts on coagulogen, a clottable protein in the lysate, producing a smaller clot protein. The clotting can be observed by turning the tube with the lysate 180° (clot end point LAL) or, in a more quantitative way, by the turbidimetric LAL, which measures kinetically ranges of the clotting. The basic principle has been improved on and modified in many ways (33). A sensitivity of 0.0005 µg/ml was determined by the developers.

The lysate is prepared by placing the crabs in restraining racks and inserting a needle through the muscular hinge between the cephalothorax and the abdominal region. Hemolymph is then drawn from the cardiac chamber into a container with anticoagulant. After collection, the amoebocytes are centrifuged and the supernatant is discarded. After 2-3 washing steps, the cells can be subjected to osmotic shock by adding distilled water and the intracellular lysate is released. The bled crabs are then thrown back into the sea, and their survival rate is unknown. In some countries (e.g. Japan) the crabs are squeezed in a mill.

One of the drawbacks of the LAL is that it only detects endotoxin (34, 35). The pyrogenic potency of non-endotoxin substances has been recognized since the 1960s, leaving a safety gap when performing pyrogen tests with the LAL. Contaminations of drugs with Gram-positive bacteria, fungi or their fragments/toxins are not an unlikely event.

1.3. Mechanism of fever

The concept of a substance produced in the mammalian organism in response to pyrogens which is causative in the genesis of fever dates back to 1948 (36). This substance, which was produced by immune cells evoked fever when injected into healthy rabbits and was then called endogenous (or

leukocytic) pyrogen. Dinarello et al. could demonstrate, that this endogenous pyrogen consisted of two distinct proteins (37), probably pro-Interleukin-1 and Interleukin-1 (IL-1). Other, similar mediators of fever were found later and were termed Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α). During a response to pyrogens, they are secreted by a subfraction of the white blood cells, the monocytes, and are called proinflammatory cytokines. It is of considerable interest that the receptors recognizing pathogen-associated molecular patterns (PAMPs) of bacteria, the so-called toll-like receptors (tlr) shares in its cytoplasmic domain the signaling areas with the IL-1 receptor (38). Additionally, all pyrogenic cytokines share a common intracellular pathway which results in the activation of the nuclear factor-κB (NF- κB). The current understanding of the mechanism of fever in the mammal is that this transcription factor results in the expression of the enzyme cyclooxygenase-2 (COX-2) which results in prostaglandin (PG) E_2 synthesis. Mice deficient in COX-2 did not develop fevers in response to LPS, IL-1, IL-6 or TNF (39-42). Specifically one of altogether four PGE_2 receptors in the brain, the EPR-3, is required to develop fever (43, 44), probably via the induction of a second messenger such as cyclic adenosinmonophosphate (cAMP) (45). That IL-1β is the most potent fever inducer compared to IL-6 and TNF-α when injected intravenously into rabbits could be demonstrated (46, 47). These findings formed the basis for the development of cell-based in vitro assays which are described in the next chapter.

1.4. Cell-based pyrogen tests

The discovery that white blood cells produce cytokines in a dose-dependent manner in response to pyrogens led to the development of altogether six in vitro assays based on primary human blood cells or cell lines. All of them have the same basic concept of incubating the substance in question at 37°C with the cells, and, as a second step, measuring the cytokine production (or, in one case, nitric oxide) by an enzyme-linked immunosorbent assay (ELISA). Four assays have been successfully validated in an international collaborative study and are described in detail in the publication of Hoffmann

et al. 2005 (48). One of these assays was the human whole blood test (IPT) whose further development is described here.

1.5. Human whole blood test (IPT)

A new way of measuring pyrogens has been introduced in 1995 by Hartung and Wendel (49). Basically, fresh heparinized human whole blood is diluted in physiological saline and brought together with the sample. In the case of pyrogenicity, the monocytes produce IL-1β in vitro over a period of 10-24 hours at 37°C which can be measured by a specific ELISA the next day. The test has a detection limit of 0.25 EU/ml and has the advantage that it is performed with the cells of the relevant species, that is, the human reaction is tested.

The ELISA (Enzyme-Linked-Immunosorbent Assay) is an assay based on the reaction of specific antibodies towards an antigen, in this case IL-1β. An antibody is bound to a microtiter plate with high protein binding capacity; the pyrogen-stimulated cell supernatant is added to the antibody and the cytokine binds. After a washing step, a second, labeled detection antibody is added which also binds to the antigen; the label is in this case biotin, which binds with high affinity to avidin coupled to POD (horseradish peroxidase). After a second washing step, substrate, in this case TMB (Tetramethylbenzidine) is added. The enzymatic reaction of the POD with the TMB changes the color of the latter from colorless to blue and the antibody-antigen reactions are made visible.

2 Aims of the study

Pyrogen testing of parenterals has been performed routinely in vivo in the rabbit since the early 1940s. Recently, a cell-based in vitro alternative has been developed which aims to replace the rabbit pyrogen test as an alternative method. The European legislation clearly states that animal testing is forbidden if there is a viable and validated in vitro alternative available. Making the human whole blood test (IPT) a standardized and commercially available alternative to the rabbit was the goal of the following work.

- The first part of this thesis validated the human whole blood test in an international collaborative study including laboratories from England, Switzerland, Norway, the Netherlands and Germany and control institutions such as the Paul-Ehrlich Institute, Germany, and the European Centre for the Validation of Alternative methods.

- The second part of this thesis standardized the most critical and the most crucial reagent: the human whole blood. In order to make this highly varying and perishable component of the assay more reliable and available, a method for cryopreserving the blood was developed, and a pooling protocol was found which levels out the interindividual differences of the human donors.

- As a third step, the whole blood test using the newly developed cryopreserved blood was validated in an international collaborative study including three different laboratories.

- The last part extended the application possibilities of the new test towards testing not hydrophilic, but lipophilic substances in order to avoid large numbers of animal experiments. The testing for pyrogens in so-called small volume perenterals, e.g. lipophilic drugs, is obligatory since January 2004 due to a change in European Pharmacopoeia.

3 Development, validation and applications of the in vitro pyrogen test (IPT) based on human whole blood

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3.1. Abstract

Microorganisms such as Gram-negative or Gram-positive bacteria, viruses and fungi contain components that activate the innate immune system. These components, called pyrogens (Greek: pyros = fire), can occur independently of viable microorganisms and are a major safety concern in parenterally administered drugs, since they can cause severe reactions such as fever, organ failure and shock in the recipient. So far, these drugs have been tested by injecting them intravenously into rabbits and measuring their fever reaction or alternatively by the Limulus Amoebocyte Lysate (LAL) test, employing the coagulation of the hemolymph lysate of *Limulus polyphemus.* Both tests have inherent limitations. A new *in vitro* test based on human whole blood, capable of

measuring all pyrogens relevant to the human patient was introduced and validated recently. This review describes its principle, development, validation and the wide spectrum of applications, such as for testing of medical devices, blood products, lipidic parenterals and air quality. This alternative method aims to replace fully the rabbit pyrogen test.

Key words

In vitro pyrogen test; interleukin-1β; validation study; alternatives to animals

3.2. Introduction

Pyrogens, as fever-inducing substances of microbial origin, can derive from dead or viable bacteria, viruses or fungi. Therefore, they can occur even in sterile environments. Contaminations of parenterals with such substances can induce local or systemic inflammatory reactions in the recipient, intended to eliminate an invading pathogen, including a rise in body temperature, but also more severe adverse reactions such as shock, disseminated coagulation, organ failure and even death. Therefore, the testing of parenterals prior to batch release is obligatory for manufacturers.

The best-known fever-inducing contaminant is a component of the cell wall of Gram-negative bacteria, i.e. endotoxin or lipopolysaccharide (LPS). Pyrogenic components of Gram-positive bacteria are equally important and include lipoteichoic acid (LTA) (26) and peptidoglycan (21 - 23). Further possible pyrogenic contaminants are exotoxins (19), enterotoxins (17, 18) , viruses (20), and fungal components (24, 25).

Classical pyrogen tests

Testing for pyrogens has been a major issue since the appearance of large volume parenterals in the 1930s. These bore a label claim of being pyrogen-free as asserted by the rabbit pyrogen test. This drew attention to the need for an official test procedure for non-pyrogenicity, which was strengthened by the heavy demand for large volume parenterals in World War II. A collaborative

study was initiated to develop the rabbit pyrogen test (10, 11), which led to the incorporation of the rabbit pyrogen test into the pharmacopoeias. Since then, all parenterals must be tested for pyrogens. This involves the measurement of the rabbit's body temperature after the application of not more than 10 ml/kg bodyweight of the substance to be tested. The very rigid fixation of the rabbit and the handling (injection procedure) can cause hyperthermia due to excitement and therefore lead to false-positive results. On the other hand, it has been reported that the fixation and lack of movement can cause a hypothermia yielding false-negative results (29).

In 1964, Levin and Bang published that the hemolymph of the horseshoe crab *Limulus polyphemus* coagulates upon contact with endotoxin. This led to the development of the Limulus amoebocyte lysate (LAL) test, which is employed to exclude endotoxin contamination in parenteral drugs (31, 32). The Limulus is collected from beaches, its hemolymph is drawn out by puncture and the animals are then thrown back into the sea. 10 to 20 percent do not survive the bleeding procedure (50-52). The mortality associated with collecting, shipping and handling the animals remains unknown. The LAL has not been able to replace fully the rabbit test, since it is defined not as a pyrogen test, but as an endotoxin test, which fails to recognize e.g. Gram-positive or fungal contaminants, toxoids, or viral antigens. Due to the crucial role of Gram-negative endotoxin, it was nevertheless possible to substitute most pharmacopoeial pyrogen testing with a mere endotoxin test. Additionally, the LAL does not reveal the biological potency of a given endotoxin in the mammal, which can differ between bacterial strains by a factor of up to 10'000 (53). Most importantly, however, certain products tested in rabbits cannot be tested in the LAL, e.g. various biologicals and vaccines, due to interference.

Fever reaction in the mammal

The finding that mammalian immune cells produce an endogenous pyrogen when in contact with pyrogenic materials dates back to 1948 (36). Bennett et al. could identify leukocytes as the source of this factor in 1953 (54). The nature of this substance was further elucidated by Dinarello et al. (37), who identified two

distinct proteins, probably the pro- and the mature form of interleukin 1β (IL-1β). The pyrogenicity of IL-1β, when injected at very low doses into rabbits, was proven by Dinarello et al. 1991 (46). IL-6 and TNF-α, which were isolated later, were found to be pyrogenic cytokines as well, though only at higher doses (46, 47).

The current understanding of the mechanism of fever in the mammal, as reviewed by Dinarello 2004 (55), is that these proinflammatory cytokines bind to receptors on the blood side of the organum vasculosum laminae terminalis (OVLT) and initiate the expression of the enzyme cyclooxygenase-2 (COX-2), which mediates prostaglandin (PG) E_2 synthesis. Mice deficient in COX-2 do not develop fever in response to injection with LPS, IL-1β or IL-6 (39-41). Specifically one of altogether four PGE_2 receptors in the brain, the EPR-3, is required to develop fever (43), probably via the induction of a second messenger such as cAMP (45). Thus, the pyrogenic cytokines cause a change in the set-point of body temperature in the hypothalamus and are therefore the mediators responsible for initiating the fever reaction. The finding that monocytes, a subfraction of the white blood cells, secrete proinflammatory cytokines such as IL-1β upon contact with pyrogenic material was the basis for the development of the whole blood test as a pyrogen test (49).

3.3. Basic principle of the whole blood test

Blood incubation

The procedure is described in detail by Hoffmann et al. (48). Briefly, freshly drawn, heparinized human whole blood from a healthy donor is diluted in physiological, pyrogen-free clinical grade saline and brought together with the test sample. In response to pyrogens, the monocytes contained in the blood sample produce proinflammatory cytokines in a dose-dependent manner. The proinflammatory cytokine IL-1β is measured by ELISA.

ELISA procedure

The IL-1β or IL-6 in the sample is sandwiched between a monoclonal coat antibody and a polyclonal peroxidase-labeled detection antibody. Unbound

material is removed by washing. The peroxidase metabolizes e.g. tetramethylbenzidine. The reaction is stopped with acid and the optical density (OD) is measured at 450 nm.

Controls

As an assay control, a dose-response curve of an LPS from *E. coli* O111: B4 is performed in parallel in each assay. This LPS is calibrated to the international WHO reference standard from *E. coli* O113: H10 (56). The dose-response curve must contain the concentration 0.5 EU/ml and a negative control. The IL-1β released in response to the concentration of 0.5 EU/ml must test positive when compared to a negative control for the experiment to be valid. 0.5 EU/ml corresponds to 50 pg/ml of the international reference standard and is considered the threshold endotoxin concentration that causes fever in the most sensitive rabbit strains. This threshold was confirmed by a study performed at the Paul-Ehrlich Institute in 2005, which analyzed 171 rabbits (16).

Testing for interference

In order to test for a given substance's interference with the activity of the monocytes, samples (pure or diluted) are incubated together with a 0.5 EU/ml concentration of the LPS dose response curve. The mean OD of the spiked sample must be within a 50-200% range of the 0.5 EU/ml concentration of the dose response curve. If this is not the case, the sample has to be diluted until the interference criteria are met.

Development of the Gram-positive standard lipoteichoic acid (LTA)

LTA from *Staphylococcus aureus* was first purified in a biologically active and endotoxin-free quality by Morath et al. (26). Later, the improved purification procedure was applied to produce LTA from *Bacillus subtilis* (57). The successful identification of the purified LTA as a pyrogenic substance, which is negative in the LAL (57) and therefore represents a pyrogenic principle that is only recognized by the rabbit pyrogen test and the cell-based assays, led to the

inclusion of the Gram-positive standard LTA derived from *B. subtilis* into the IPT procedure.
In order to make this method commercially available and replace the rabbit pyrogen test, the following steps were taken:

3.4. Comparison of the in vitro reaction of human whole blood with that of rabbit whole blood

Since the human whole blood test (WBT) aims to replace the rabbit pyrogen test, the sensitivity of both species towards different pyrogenic stimuli was compared using human and rabbit whole blood. For this, a rabbit whole blood test was developed which followed the procedure of the human whole blood incubation in every detail (58). Overall, the IL-1β response of the rabbits towards different pyrogenic stimuli was comparable to that of humans. In the case of the Gram-positive stimulus, LTA, the rabbit blood was less sensitive than human blood, thus confirming the human whole blood test as an equal or even superior test system to reflect the human response.

3.5. Establishment of the IPT as a test for biologicals

Biologicals, such as protein solutions, cytokines, antibodies, heat shock proteins, blood coagulation factors and vaccines for intravenous use, pose a particular problem in pyrogen testing. They can influence the LAL results due to their characteristics, such as color and viscosity, and they are potentially immunogenic in the rabbit, causing fever reactions that are independent of contaminations. In any case, if immunogenic substances are tested, the animals may only be used once, which results in extremely high costs for the manufacturers. The IPT does not pose such problems. Some examples of the application of the IPT for pyrogen testing of such samples are given below.

Control of vaccines

In 2001, new batches of vaccines against early summer meningoencephalitis were released that caused severe fever reactions in some recipients. Although they were negative in the LAL, these batches gave a high signal in the WBT.

This phenomenon could be shown to be due to the removal of the mercury-containing additive thiomersal. The additive suppressed the IL-1β response in the WBT, making it likely that it also suppressed the pyrogenic property of the attenuated virus in the vaccine in earlier batches (59).

In 2003, Carlin and Viitanen demonstrated that trivalent vaccines (diphtheria, tetanus and polio), which tested negative in the LAL, were powerful inducers of IL-6 in 4 out of 8 donors in the whole blood incubation (60). This difference between the LAL and the WBT could be attributed to the toxoid of *Corynebacterium diphteriae,* and, to a lesser extent, to that of *Clostridium tetani*, both non-endotoxin pyrogens. (61). Additionally, in both studies, the authors found pronounced differences in the IL-6 and IL-1β response of different donors towards the vaccines and their components, although they displayed highly conserved LPS reactivity. This indicated a more variable interindividual sensitivity of human donors towards these non-endotoxin stimuli. Nonetheless, it was demonstrated that pyrogenic reactions towards non-endotoxin stimuli can be just as vigorous as those towards endotoxin. These results show that the rabbit pyrogen test cannot be replaced by the LAL for vaccines, but that only the measurement of the cytokine response of primary human cells, e.g. the WBT, represents an adequate alternative.

Measurement of albumins

Pyrogenic reactions of human patients after the administration of human serum albumin, which had tested negative in the rabbit, were observed in 1978 (62). In this study, the LAL yielded positive results without perceivable patient reactions. Pool et al. (63) tested 22 batches of human serum albumin (HSA), fibronectin and stabilized human serum solutions using artificial contaminations of endotoxin and LTA from *B. subtilis*. None of these products interfered with the production of IL-6 by whole blood, whereas one batch of artificially contaminated albumins tested false-negative in the LAL. Another study using the WBT performed with albumins, coagulation factor, vaccines and immunoglobulins indicated a high sensitivity and reliability of the WBT for these substances (19).

A comparison between the rabbit and the human whole blood test for the detection of pyrogens in albumins was performed by Spreitzer et al. (64) with 29 batches of human serum albumin. The WBT was clearly superior to the rabbit test, especially at the limit of detection of 5 EU/kg (0.5 EU/10ml/kg), with the WBT retrieving all 29 spiked samples as positive compared to only 5 positive rabbit tests and 23 temperature rises, which would have required a repetition of the test. This limit of detection represents the 0.5 EU/ml pyrogenic threshold.

3.6. Validation

Six cell-based assays, including two variants of the WBT measuring IL-1 and IL-6, respectively, were validated in an international collaborative study including laboratories from Austria, Germany, Switzerland, England, Norway and Italy and the participation of control institutions. The study validated assays such as the cell line THP-1 with the endpoint TNF-α (65) or with the endpoint neopterin (66, 67), the cell line Monomac-6 measuring IL-6 (68), isolated peripheral blood mononuclear cells (PBMCs) with endpoint IL-6, and the human whole blood test (49), using blinded endotoxin stimuli and altogether 13 intravenously applied drugs. Sensitivities ranged between 73-96% and specificities between 90-97%.

The WBT measuring IL-1 achieved 73 and 93%, and the WBT measuring IL-6 88.9 and 96.6%, respectively. The development and outcome of this study is described in detail elsewhere (48, 49, 69- 71)

3.7. Development of the commercially available IPT kit

The established WBT procedure was adapted to materials provided by Charles River Endosafe and a commercial kit was developed, which was named In Vitro Pyrogen Test (IPT). This kit contains all the reagents necessary for the incubation and ELISA procedure except for the human whole blood.

Development of cryopreserved blood

Fresh human whole blood is a highly perishable item that cannot be stored longer than 4 hours at room temperature without loss of sensitivity. Additionally,

it is not easily available, a potential hazard due to unrecognized infections (HIV, hepatitis) and, due to donor differences, cannot be standardized. In order to overcome these difficulties a procedure was developed to successfully freeze and store whole blood. The protocol closely followed the method of de Boer, 1981, who had already successfully frozen isolated monocytes (72). Blood from five healthy donors is mixed with 10% endotoxin-free dimethylsulfoxide (DMSO) (v/v ratio) and left to stand for 15 minutes. The blood is then pooled (Fig. 1) and frozen in a computer-controlled freezing process to –120°C. The blood is stored in the vapor phase of liquid nitrogen and, after thawing, can be used like fresh blood without any washing steps. The cryopreserved pooled blood renders highly reproducible results and is at least equal to fresh blood concerning a wide variety of applications and stimuli (73).

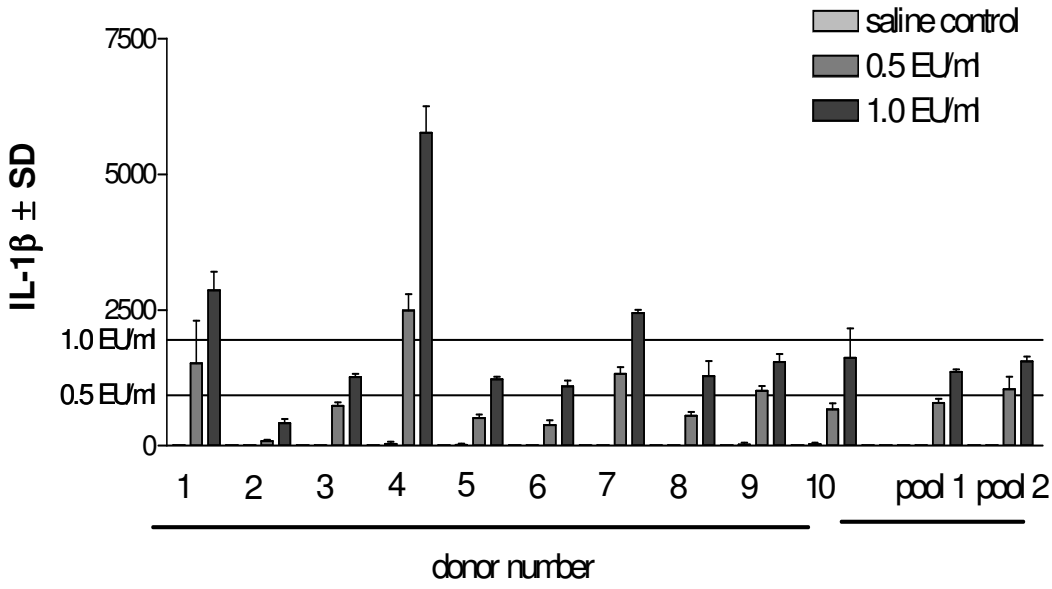

Fig. 1: Comparison of the reactivity of frozen blood from 10 individual donors and that of pooled blood from the same donors.

The calculated means of the response of the five individual donors towards the 0.5 EU/ml LPS corresponds to the response of the pooled blood. The higher response of donor 4 is therefore leveled out.

Pool 1: The blood was pooled before freezing

Pool 2: The blood was pooled after thawing

Since blood frozen using the described method could only be stored and shipped in the vapor phase of liquid nitrogen, a reagent that is not available to all laboratories, an alternative freezing method was developed by the Paul-Ehrlich Institute, Langen, Germany. The method is described in detail by Schindler et al, 2006 (74). The alternative cryopreservation method provided blood that could be stored (and shipped) at -80°C, therefore making the blood available for users without liquid nitrogen infrastructure.

Validation of the cryopreserved blood

In an additional validation process, which followed the exact procedure of the former process described above, both methods of cryopreservation were validated (74). Furthermore, the IPT incubation steps, which had been developed and validated in pyrogen-free reaction tubes, had in the meantime been successfully transferred to the 96-well microtiter plate by reducing the volumes used and adapting the protocol accordingly. Therefore, the fresh blood incubation in the microtitre plate was validated as well as the cryopreserved blood both in the 96-well microtiter plate and in the pyrogen-free reaction tubes. The overall performance of all approaches was very good, with sensitivities of over 90% and specificities around 80%. Remarkably, these excellent performance characteristics were achieved although the spike concentrations chosen were at or below the defined pyrogenicity threshold of 0.5 EU/ml (48). Indeed, the few misclassifications only occurred for these borderline cases. Therefore, the IPT could be improved concerning its availability, its performance and its handling (Table I).

Test	Inter-laboratory reproducibility (%)	Sample size: sensitivity	Sensitivity (%)	Sample size: specificity	Specificity (%)
WBT Fresh blood Reaction tubes	DL-NL1: 72.9 DL-NL2: 81.6 NL1-NL2: 70.2	88	72.7	59	93.2

IPT Cryopreserved blood (-80°C)	DL-NL 1: 86.7 DL-NL 2: 87.5 NL 1-NL 2: 100	77	97.4	45	82.2
IPT Cryopreserved blood (nitrogen)	DL-NL 1: 66.0 DL-NL 2: 63.3 NL 1-NL 2: 83.3	74	82.4	46	89.1
IPT Fresh blood plate	DL-NL 1: 88.1 DL-NL 2: 89.7 NL 1-NL 2: 91.5	84	98.8	55	83.6

Table I: Outcome of the validation of the basic WBT procedure using reaction tubes and fresh blood and of the IPT methods using cryopreserved or fresh blood in a microtiter plate.

3.8. Special adaptations

Medical devices

Due to manufacturing and handling, medical devices can bear pyrogens on their surface which, when brought into the human organism may lead to inflammatory reactions and reduced biocompatibility. Recognizing this problem, the Medical Device Directive 93/42 EEC states that medical devices must be designed and manufactured in such a way that they will not compromise the clinical condition or the safety of the patients. The Association for the Advancement of Medical Instrumentation (AAMI) stated in 2001 that products with direct or indirect contact with the circulatory system or the lymph or products that interact systemically with the body should be tested for pyrogens (75).

Products in direct (blood bags, needles) and indirect (swabs, gloves) contact to the blood circulation can have a serious impact on the organism, as contaminations induce systemic reactions. A severe case of contact dermatitis due to endotoxin contamination of surgical gloves was described in 1984 by

Shmunes and Darby (76). After eight pyrogenic reactions in 69 patients undergoing heart catheterization, Kure et al. described endotoxin contamination of extracts of the hospital's surgeon's latex gloves, which evoked fever in rabbits and could be successfully transmitted to cardiac catheters (77). Grötsch et al. were able to evoke fever reactions in rabbits with an eluate of gloves found to contain up to 2560 EU (78).

Medical devices pose a particular problem for pyrogen testing, since they cannot be examined directly with the rabbit or the LAL test. Their diversity with regard to size, form, material and form of application challenges the existing assays, demanding individual approaches. 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. However, it is unclear, how well rinsing a medical device in water can release pyrogens from its surface and the dilution of such released pyrogens in a large volume of rinsing water reduces the limit of detection. The alternative of transplanting the questionable material directly into the rabbit is highly invasive, causing possible reactions not associated with pyrogenic contaminations but rather with tissue damage and is therefore questionable in its ethical and scientific implications. The obvious advantage of the IPT over the classical test methods is that the whole blood comes into direct contact with the respective device and no preparation of an eluate is required. This has been demonstrated using aneurysm clips as proof of principle (79). Additionally, unlike the LAL, the IPT detects all pyrogens relevant to humans, not only endotoxin.

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 80, 81). Its specific preference for rough and hydrophobic surfaces differs from that of fibroblasts (82). The role of cytokine production of the monocytes/macrophages in the early stages of implant insertion is poorly understood. The fact that some materials are obviously capable of modulating the cytokine response (83, 84) makes it difficult to distinguish a genuine pyrogenic contamination from an unspecific activation

and poses the problem of adequate negative controls. For this purpose, a process was developed for the thorough depyrogenation and a device was developed for the testing of metallic or plastic surfaces with the IPT in order to gain information about possible inherent activating or inhibiting characteristics of materials (85). The device was made up of a perforated metal plate pressed onto the sample surface by screws through a metal frame. The resulting wells were watertight due to the use of washers. The blood was incubated directly in the wells of the depyrogenized device contacting the surface to be tested. The study showed that pyrogenic contaminations on surfaces could be reliably removed only when heated for 5 h at 300 °C. This applied to titanium, titanium alloy ($TiAl_6V_4$) and steel material for implants. Artificial contaminations were detected in a dose-dependent manner.

Some medical devices are absorbed completely by the body, as are any contained pyrogenic contaminations. Examples are liposomes and alginate microcapsules used as drug carriers. The detection of pyrogenic contaminations in alginate microcapsules is illustrated in Fig. 2.

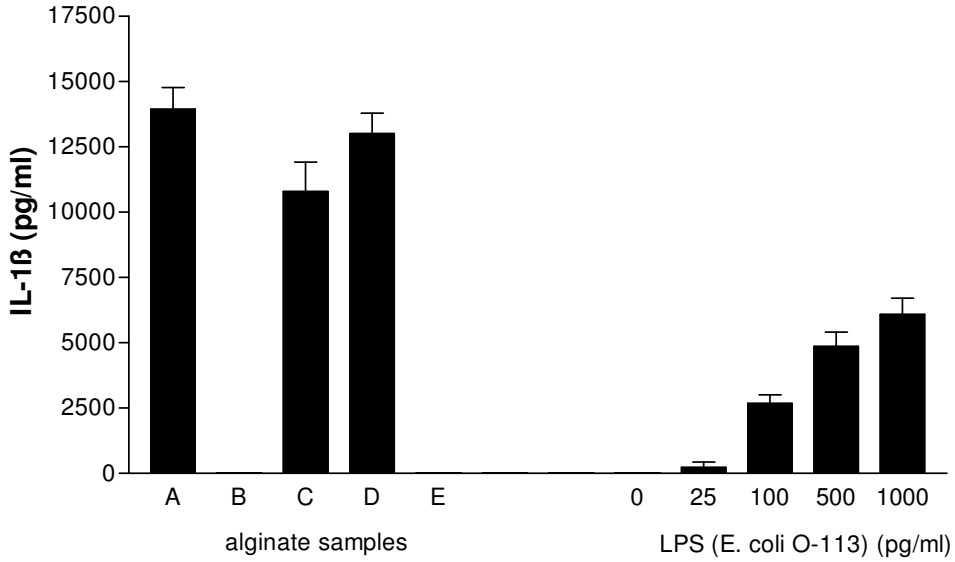

Fig. 2: IL-1β production of fresh blood upon stimulation with different alginate solution samples (A-E).

Cellular therapeutics

Cellular therapeutics are defined as living cells that are transferred into the intact organism in order to introduce a new or restitute a defective function (86). This includes a wide variety of cells such as chondrocytes, stem cells, bone marrow cells, and blood cells such as lymphocytes, erythrocytes, thrombocytes. The latter pose a particular problem, since they are stored at room temperature and are therefore easily subject to extensive bacterial growth. Transfusion reactions may range from shivering, fever and chills all the way to septic shock. The problem is rather under- than overrated, since numerous clinical events are not recognized as being transfusion-associated, but are often rather attributed to the underlying disease. Additionally, medication and immunosuppression might mask an existing septic/pyrogenic event which likely contributes significantly to the patient's overall morbidity. Recently attention has focused on viral infections, although the incidence of viral contaminations of blood products is less than 1 in 1.000.000 per unit for HIV in comparison to 1 in 3000 for bacterial contaminations (87).

Two large studies in France (BACTHEM study, 88) and the USA (BaCon study, 89) revealed that platelets hold a significantly higher risk of bacterial contamination than red blood cells, irrespective of whether they were single-donor or pooled preparations. Pathogens associated with bacteremia in the US study were 59% Gram-positive (mainly skin contaminants such as staphylococci, streptococci and propionibacteria) and 41 % Gram-negative (coli, serratia, enterobacter). Gram-negative *Yersinia enterocolitica* was not found in that study, although it occurs frequently in transfusion-related sepsis and was responsible for 7 of the 8 fatalities recorded in the US between 1986 and 91.

Incidences of microbial contamination increased with prolonged storage, and both studies linked fatalities to the occurrence of Gram-negative bacteria. The US study also determined endotoxin levels (up to 273,500 EU/ml, according to LAL). The authors estimated rates of transfusion-transmitted bacterial infections of 1:100'000 for platelets and 1 in 5 million for red blood cells, with fatalities of 1 in 500.000 and 1 in 8 million, respectively. Overall, Gram-negative bacteria

tended to occur more frequently in red blood cells, probably due to the storage at lower temperatures.

In 2004, a roundtable meeting on bacterial detection took place during the Annual Congress of the International Society of Blood Transfusion in Edinburgh to discuss the limitations of current testing methods. Currently, culturing methods, such as the very sensitive BacT/ALERT method, are considered the best, though they are very time-consuming (12 h to 7 days). Platelet concentrates are released on a “negative-to-date” base and recalled if necessary. The panel reported occurrences in the Netherlands, where platelet concentrates containing skin bacteria were tested positive only after 48 h. By this time, the batch had been released and about 50% of the units had already been transfused. Very similar events were described by Belgian blood centers (87). Anaerobic bacteria, e.g. *Corynebacterium* spp., are picked up even later and there is extra cost involved. However, anaerobic bacteria have been linked to fatal septic transfusion incidents (90). Altogether, culture methods are incapable of providing complete safety, and other, especially quicker methods, are sought.

A method to inactive contaminating bacteria in transfusion products by photochemical treatment (PCT) (91) has been developed. Still, it must be kept in mind that although this inactivation may inhibit growth, it will have no influence on the already existing pyrogenic content. Therefore, the testing of these cellular products and their suspension materials is an interesting future challenge for the IPT. Pretesting of clinical grade erythrocytes and thrombocytes intended for transfusion indicated interference-free retrieval of an artificial endotoxin spike (Fig. 3) when compared to the saline control.

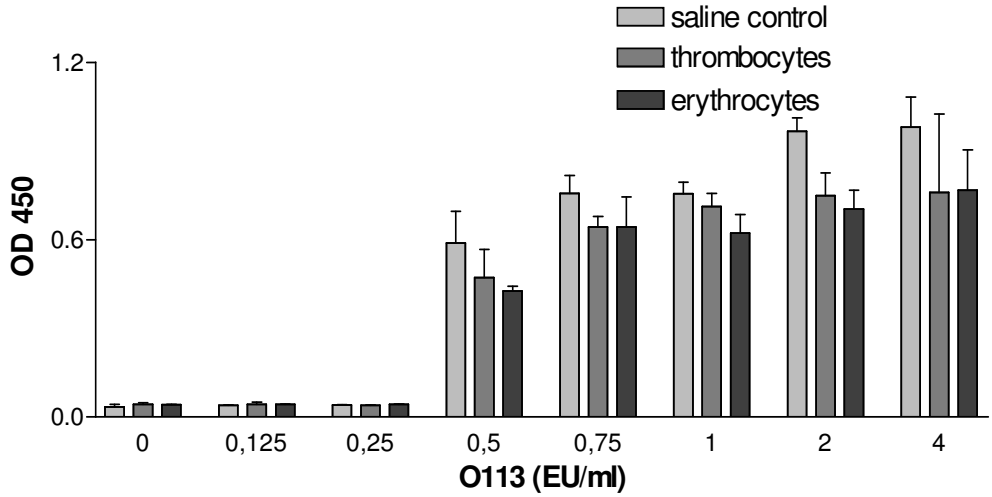

Fig. 3: Retrieval of endotoxin spikes in red blood cell and platelet concentrates

Dialysis

Pyrogenic reactions in hemodialysis patients at the end of a session were first associated with high bacterial and endotoxin levels by Raij et al. (92) and Favero et al. (93). Since then, contaminations have been found in the pure water (94-97), the machines, especially in areas with low circulation or dead spaces which serve as a reservoir for bacteria (98), filter materials (99) and bicarbonate concentrates (95).

In 1993, the AAMI 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 (97), Greece (100), the USA (94), and Canada (101) 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 400 l of dialysis fluid a week. Next to Gram-negative bacteria, 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. That this might indeed be crucial for judging the pyrogenic exposure of a dialysis patient was assessed by

Marion-Ferey et al., who tested scrapings of bacterial biofilms in dialysis tubes and found a 20-fold higher response in the IPT than in the LAL (102). The passage of cytokine-inducing substances, not only endotoxins, but exotoxins and peptidoglycans as well, through the dialysis membrane has been demonstrated (103- 106).

The chronic exposure of dialysis patients to even low concentrations of pyrogens is thought to contribute to inflammatory processes in the joints and bones and therefore to the carpal tunnel syndrome and arthropathy associated with long-term hemodialysis (107, 108). In 1991, Baz et al. showed that the use of ultrapure water delays the onset of the carpal tunnel syndrome (109). The group of Schwalbe (110) showed in a retrospective study that the incidence of amyloidosis decreased between 1988 and 1996 along with 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 with pyrogen exposure has yet to be established.

In all, the testing for pyrogens in dialysis fluids is a crucial issue for the safety of the patients. Since the fluids themselves are either highly hyper- or hypotonic, a variant for testing dialysis fluids in the IPT established the percentages of diluents and samples that can be tested (own unpublished results). Still, the problem remains that the patients receive very high volumes of fluid in one session, and therefore pyrogens must be detected at very low concentrations. A promising possibility is a modification of the basic IPT protocol, the so-called adsorb and wash IPT (AWIPT), discussed later, which can concentrate pyrogens on the surface of albumin-coated macroporous Matisse™beads, thus enhancing the sensitivity by a factor of 250 (111).

Airborne pyrogens

Inhalable whole or fragments of microorganisms have long been recognized as causes of airway hyperreactivity. Monday sickness with its typical symptoms (chest tightness, respiratory distress and coughing) was described as early as 1936 (112). In 1942, rural mattress makers experienced headache, nausea,

chills and fever about 6 hours after exposure to low grade cotton. Neal et al. associated these phenomena with high numbers of a Gram-negative bacterium in the material (113). Additionally, milder symptoms occurring 8 hours after exposure could also be evoked by sterilized cotton, which was thought due to remaining endotoxin. A highly significant ($r > 0.95$) dose-response relationship between Gram-negative bacterial count and symptoms of byssinosis such as chest tightness, airway inflammation and coughs was established by Cinkotai et al. (114). In the same study, a good correlation existed between symptoms and mainly Gram-positive bacteria, whereas one to fungal spores could not be established. Acute bronchoconstriction as well as chronic airway irritation with bronchitis and decrements in airflow over the work day have been reported for personnel working in animal confinement buildings (115 - 117). Long term consequences are of allergic, inflammatory and immunostimulatory nature, e.g. organic dust toxic syndrome (ODTS) and chronic bronchitis. The LAL test for these contaminants has the drawback that it does not reflect the biological potency of a given LPS in the mammal (53) and the LAL test can only be performed with an eluate of a filter or by impingement, i.e. the air to be tested is led through pyrogen-free water which is then tested in the LAL. The higher pyrogen retrieval by impingement when compared to filtration, possibly due to the incomplete eluation of the sample from the filters, was demonstrated by Zucker et al. (118).

A new approach of measuring the integral inflammatory activity in air samples in different environments by IPT was reported by Kindinger et al., 2005 (119). A defined amount of air is drawn through a filter in a sealable plastic monitor. The blood incubation is performed directly on the filter inside the monitor, thus making any handling of the filter unnecessary. When compared to the LAL, a 2-25 fold higher pyrogenic load was found in the IPT in samples drawn in parallel. Epidemiological studies will show what levels of exposure to inflammatory stimuli in the air eventually lead to the above-mentioned lung diseases.

Lipidic formulations

In January 2004, pyrogen testing of so-called small volume parenterals (< 15ml) became obligatory in Europe. This concerns many formulations that had not been subjected to pyrogen testing before, such as vitamin preparations and steroids. Many of these are applied intramuscularly or subcutaneously and are therefore not necessarily of a hydrophilic nature. This poses a completely new challenge to all methods of pyrogen testing, since a lipophilic substance on the one hand cannot be injected intravenously into the rabbit due to the danger of clogging small vessels with lipid drops and severely damaging vital organs and will, on the other hand, influence the optical density measured as the readout in the LAL due to the formation of oil droplets. Furthermore, the pyrogenic portion of LPS, lipid A (for review see Rietschel et al., 1993 (120) has been reported to be masked by lipoproteins (121) and lipophilic parenterals (122) in the LAL.

Therefore, the IPT procedure was adapted to suit lipophilic substances. 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 interfered strongly by suppressing the endotoxin stimulus added. Oils that proved interference-free were then used as diluents for interfering end-products. It was possible to dilute their 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 (1 ml per person), a relatively high endotoxin concentration can be tolerated. The established protocol leaves a broad safety margin, especially since the strict criteria for intravenous drugs were applied to this situation (123).

AWIPT (absorb and wash IPT)

Another interesting development is the so-called absorb and wash IPT (AWIPT). It uses porous acrylic beads with immobilized albumin, which has a higher affinity than native plasma albumin to endotoxin (124), to separate the

pyrogenic contamination from the sample. These beads were originally developed as LPS adsorbers (Matissebeads™) to be applied in sepsis patients. The AWIPT uses this material to collect the endotoxin contained in a sample after an absorption phase in the substance to be tested. The beads are then washed in order to remove the unbound material and can then be used directly in the IPT incubation. It could be shown that this works also for LTA of *Staphylococcus aureus* and zymosan, a yeast extract. This procedure has already brought promising results with substances that interfere with the standard IPT procedure, i.e. toxic or immunomodulatory drugs (111). Another possibility is the testing of high-volume parenterals such as dialysis fluids, which contain endotoxin concentrations below the detection limit of other pyrogen tests. The beads could be used to concentrate the endotoxin on their surface from a large sample volume. Using this procedure, the detection limit of the IPT could be lowered from 0.25 EU/ml of *E. coli* endotoxin down to 1 x 10^{-5} EU/ml (Fig. 4).

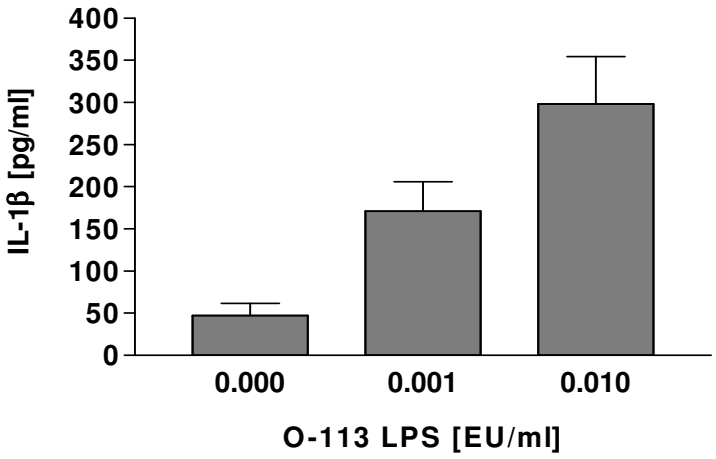

Fig. 4: Limit of detection in the AWIPT

In all, the further development of the IPT into the modified form of the AWIPT promises to overcome shortcomings for special applications caused by interferences of certain drugs or substances with the classical IPT procedure. It allows lowering of the detection limit, and provides a useful tool for the testing of toxic or strongly interfering substances, even those that suppress the immune system and therefore cytokine production.

3.9. Conclusions

Pyrogens (fever-inducing substances) from microorganisms can occur as contaminations of parenterals. Until now, the safety of injectable drugs has been assessed by the *in vivo* rabbit pyrogen test and the *in vitro* Limulus amoebocyte lysate test (LAL).

The new cell-based *in vitro* pyrogen test based on fresh or cryopreserved human whole blood (IPT) has been successfully validated and has proven to be a reliable and useful tool for a wide spectrum of applications, ensuring patient safety in many medical fields such as hydrophilic and lipophilic drugs, dialysis fluids, airborne pyrogens, medical devices and biologicals. It is capable of measuring all known pyrogens relevant for the human and is highly reliable, robust and easy to perform.

3.10. Appendix

Abbreviations: AAMI, Association for the Advancement of Medical Instrumentation; AWIPT, absorb and wash in vitro pyrogen test; cAMP, cyclic adenosinmonophosphate; DMSO, dimethylsulfoxide; ECVAM, European Centre for the Validation of Alternative Methods; ELISA, enzyme-linked immunosorbent assay; ELC, endotoxin limit concentration; EU, endotoxin unit; HD, hemodialysis; HSA, human serum albumin; IL, interleukin; IPT, in vitro pyrogen test; LAL, Limulus amoebocyte lysate; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NIH, National Institutes of Health; NIBSC, National Institute of Biological Standards and Controls; OD, optical density; OVLT, organum vasculosum laminae terminalis; PBMCs, peripheral blood mononuclear cells;
PEI, Paul-Ehrlich Institute; PG, prostaglandin; POD, peroxidase; RNA, ribonucleic acid; TMB, tetramethylbenzidine; TNF, tumor necrosis factor; USP, United States Pharmacopoeia; WBT whole blood test

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4 International validation of novel pyrogen tests based on human monocytoid cells

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4.1. Abstract

Parenteral medicines are required to be tested for pyrogens (fever-causing agents) in one of two animal-based tests: the rabbit pyrogen test and the bacterial endotoxin test. Understanding of the human fever reaction has led to novel non-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. The here 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

Abbreviations: BET, bacterial endotoxins test; CI, confidence interval; DL, developing laboratory; ELC, endotoxin limit concentration; ELISA, enzyme-

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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 mononuclear blood cell; PBS, phosphate buffered saline; TLR, toll-like receptor; TNFα, tumor necrosis factor α; WBT, whole blood test

4.2. Introduction

Pyrogens, a chemically heterogeneous group of fever-inducing compounds, are derived from bacteria, viruses, fungi or the host himself. Monocytes/macrophages react to microbial products during an immune response by producing endogenous pyrogens such as prostaglandins and the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) (132). 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., for more than sixty years, the rabbit pyrogen test or the bacterial endotoxins test (BET), often referred to as 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 alone 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 (125) that was implemented e.g. into European legislation in 1986 (126), 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 comprised largely of lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria that stimulates monocytes/macrophages via interaction with CD14 and toll-like receptor 4 (TLR4) (127) is the pyrogen of major concern to the pharmaceutical industry due to its ubiquitous sources, its stability and its high pyrogenicity (128-130). 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 (31). In the United States, Limulus crabs are generally released into nature 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 of the horseshoe crab population such as its 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 (53,131). 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 (132), 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 (133, 134). Subsequently, a number of different test systems, using either whole blood, PBMC or the monocytoid cell lines MONO MAC 6 (MM6) (68) or THP-1 (135) as a source for human monocytes and various read-outs have been established and were recently reviewed (136). 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 and allowing their 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 (137-139).

4.3. Methods

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, 20 EU in 1 ml/kg of E. coli LPS (EC5) (140) or EC6 (56) 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 (141).

In vitro monocyte-based tests

Good laboratory practice concordant Standard Operating Procedures of the various methods were made available by ECVAM (www.ecvam.jrc.it). The test systems are summarized by Hartung et al. (70) and detailed in previous work (49, 66, 67, 69, 142, 143).

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 (56). Test materials for validation are specified in the Results section. All consumables were purchased as sterile and pyrogen-free and not specified reagents were pro analysis grade.

PBMC-IL6

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 to be taking drugs known to influence the production of cytokines. Using a heparinized (50 µl Fragmin at 10000 IU, Dalteparin, Pharmacia) syringe, 30 ml blood were collected. Within two hours, PBMCs were isolated from 20 ml Lymphoprep (Nycomed, Oslo, Norway), 15 ml PBS and 15 ml of heparinized whole blood by centrifuging at 340 x g for 45 min at room temperature. The PBMC-layer was washed twice with PBS centrifuging at 340 x 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 shall be incubated with samples within four hours after blood withdrawal.

Protocol for PBMC-IL6

In quadruplicate per 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 a 96-well tissue culture plate (Falcon Microtest, Becton Dickinson Labware) at

37°C for 16- 24 hours in an atmosphere of 5% CO_2 in humidified air. After incubation, 50 µl of supernatant from each of the wells was transferred on the ELISA plate ensuring that cells are not aspirated by angling the assay plate.

ELISA for PBMC-IL6

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 microtitre plate (Nunc-Immuno 96-well plate MaxiSorp, F96; Life Technologies™) at 15 - 25 °C for 16 - 24 hours. 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 albumine). Plates were incubated with 200 µg/ml horseradish peroxidase conjugated to sheep anti-IL-6 antibodies (Novartis, in-house) for 2-3 hours at 20-25°C. Shortly before use, 90 ml substrate buffer and 4.5 ml TMB solution (240 mg 3,3',5,5'Tetramethyl-benzidine 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 minutes, the enzyme reaction was stopped by 50 µl of 5.4% H_2SO_4 per well. The absorbance was measured at 450 nm using 540-590 nm as reference wavelength.

WBT-IL1

Blood Collection for WBT-IL1

Blood donors should show no evidence of disease or need of 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 four hours (144).

Protocol for WBT-IL1

In this order and in quadruplicates 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°C ± 1°C for 10-24 hours. The incubation tubes were mixed thoroughly by inverting them. Incubations were centrifuged for 2 minutes at 10.000 g and the clear supernatant, taking aliquots of ≥ 150 µl, was used for the ELISA (ENDOSAFE-IPT, Charles-River Endosafe, Charleston, USA) following the manufacturer's procedure.

WBT-IL6

Blood Collection for WBT-IL6

Blood donors were selected as described for PBMC-IL6. 30 ml 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 four hours (174).

Protocol for WBT-IL6

In quadruplicate per 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 a 96-well tissue culture plate (Falcon Microtest, Becton Dickinson Labware) at 37°C for 16-24 hours in a humid atmosphere of 5% CO_2. After incubation, 50 µl of supernatant from each of the wells was transferred on the ELISA plate ensuring that cells are not aspirated by angling the assay plate. The same IL-6 ELISA as for PBMC-IL6 was used.

MM6-IL6

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) (e.g. Life Technologies™) added and then centrifuged at 100 x 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 non-essential 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 x 10^5 cells/ml twice a week.

Protocol for MM6-IL6

To pre-incubate the cells for a test, 30-50 ml of cell suspension were centrifuged at 100 x 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 4x10^5 cells/ml. The cells were incubated approximately 24 hours at 37°C, 5% CO_2 and high humidity. Cells were washed and counted as above, diluting to 2.5 x 10^6 viable cells/ml, just prior to addition to the culture plate. In quadruplicates, 50 µl of samples/controls, 100 µl of RPMI-C and 100 µl of gently swirled MM6 were incubated in 96-wells tissue culture plates at 37°C for 16-24 hours with 5% CO_2 and humidified air. After incubation, 50 µl of supernatant from each of the wells was transferred on the
ELISA plate ensuring that cells are not aspirated by angling the assay plate. The same IL-6 ELISA as for PBMC-IL6 was used.

THP-Neo

Cell culture for THP-Neo

THP-1 cells were obtained from the American Type Culture Collection (ATCC, TIB-202). 6 x 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 upright position at 37° C with 5% CO_2 and humidified air. On the fourth day of culture, further 30 to 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 have to be grown for two to three weeks in order to ensure that they divide properly before using them for tests. Furthermore, cells should not be kept in culture for more than four months but new cultures should be 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 x 10^7 cells (for one plate) were centrifuged at 400 x g and 20° C for 7 min and resuspended in 20 ml medium, 2 mM L-glutamine and 50 µM 2-mercaptoethanol.

Protocol for THP-Neo

100 µl 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. 200 µl/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

hours 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.

THP-TNF

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 aminoacids 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 are 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 cultivating 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 hours. Cells were collected, centrifuged and resuspended in culture medium containing calcitriol (final concentration 100 ng/ml). They were counted and adjusted to 1 to $1.25x10^6$ cells/ml. Cells were cultured for 44-48 hours in T25 flasks. Then, terminally differentiated cells were harvested and counted using a haematocytometer and trypan blue. Cells were diluted to $1.25x10^6$ cells/ml and 200 µl of suspension were dispensed into each well of the above 96-well cell culture plate containing already 50 µl of sample/control in quadruplicates. Plates were incubated for 16-24 hours at 37°C and 5%CO_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 ºC overnight. 50 µl of sample/control (in quadruplicates) or duplicates of TNFα standards (250, 62.5, 15.6, 3.9, 0.98, 0.24, 0 U/ml, NIBSC) were added for 16-24 hours at 37°C and 5% CO_2. An

aliquot of the detecting antibody (biotinylated goat-anti-human TNF-α from the Duoset kit, R&D) 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). 100 µl were dispensed to each well for two hours at room temperatue. After washing, 100 µl Streptavidin-peroxidase conjugate (R&D) was added for 20 min. After washing, 100 µl of TMB (Sigma) were dispensed and incubated in the dark before reading at 650 nm. Incubation time was chosen so that 250 U/ml TNFα value had an OD $\geq$ 1.5.

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. In case of the within-laboratory reproducibility, where three independent but identical runs were performed, the mean similarity was calculated.

A one-sided t-test, assuming hazard and thus designed to proof 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 compares the data of a given sample against the data of the standard positive control of 0.5 EU/ml, which is performed in parallel. It is 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 otherwise. This means that a negative sample had to be significantly lower than 0.5 EU/ml. The levels of contaminations 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 2x2-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 (145).

4.4. Results

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 EU/ml (Fig. 1). This approach was recently described in more detail and further exploited (16). The model indicated that 50 % of the animals develop 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 of 4.24 to 6.21 EU/ml. Only at 20 EU per kg of body weight, all animals showed 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 per 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.

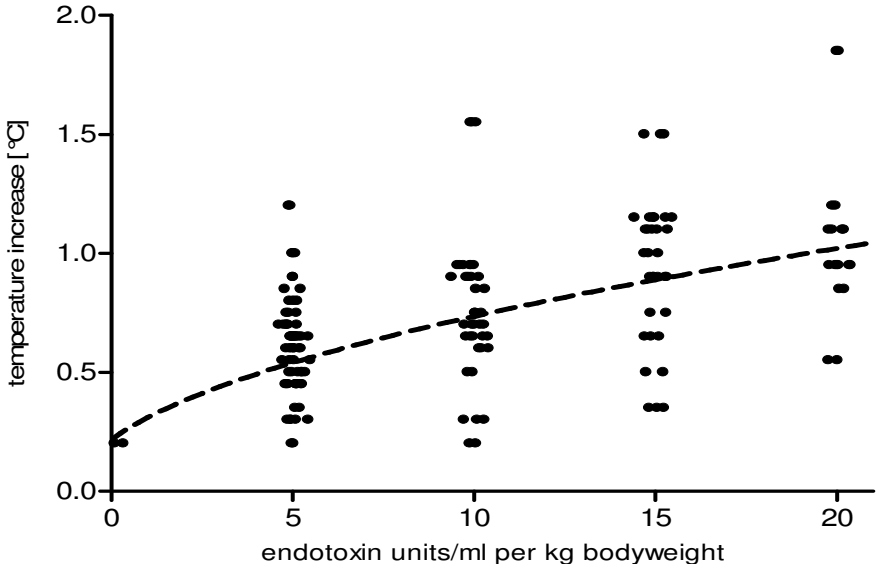

Fig. 1. Temperature increase of 171 rabbits upon endotoxin injection with a fitted regression line

The maximum temperature increase in ºC within 180 minutes after endotoxin injection of 171 rabbits is presented. The mean temperature increase, modeled with regression techniques, is indicated by the dotted line.

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 proved 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).

Test	System	Readout	Ref.	Within-laboratory reproducibility (%)	Between-laboratory reproducibility (%)	Sensitivity (%)	Specificity (%)
WBT-IL6	whole blood	IL-6	136	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	49	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	143	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 (68)	IL-6	136	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	65	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 (135)	neo-pterin	65	DL: 100 NL1: 94.4 NL2: 77.7	DL-NL1: 97.2 DL-NL2: 50.0 NL1-NL2: 51.8	88.9	72.2

Table 1: Novel pyrogen tests and their performance in prevalidation

Protocols for all methods are listed in Poole et al. (136) and in the Methods section. All tests include dilution of the sample by 1:5 with the exception of the IPT-IL-1 test that requires a 1:12 dilution of the sample. The WBT-IL6 and the PBMC-IL6 tests combine data from three respectively four blood-donors per run, the WBT-IL1 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 besides WBT-IL1 (100 samples). Sensitivity describes the probability to correctly classify positive samples and specificity describes the probability to correctly classify negative samples.

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

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 (146), 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.

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	metoclo-pramid	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	dimetinden-maleat	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

Table 2: Test substances for the validation phase

* 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.

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 (146).

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 classification 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 conducted by which the likelihood of underestimation of pyrogen content is maximized and thus possible negative consequences for health can be estimated.

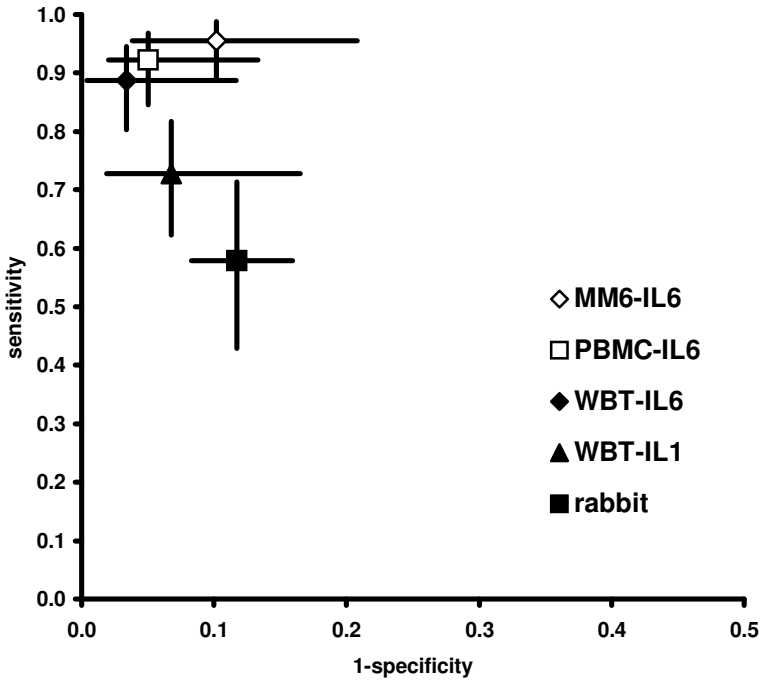

Fig. 2. Sensitivity and specificity of four in vitro assays in the validation study and modeled rabbit test performance with 95%-confidence intervals

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. 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 and the multiple-donor approach used for the other tests that is more conservative and laborious, but decreases the probability for false-negative classification. 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 pyrogen tests listed in Table 3 show parameters of performance outperforming the rabbit pyrogen test.

Test	Between-laboratory reproducibility	Sample size: sensitivity[#]	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[†]	-	-	57.9	-	88.3

Table 3: Validation of the predictive capability of novel pyrogen tests

[#] sample sizes are reduced by outlier exclusion defined in the study protocol

[†] parameters calculated by the fitted regression model

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 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-NI even resulted in the maximum sum of 2.

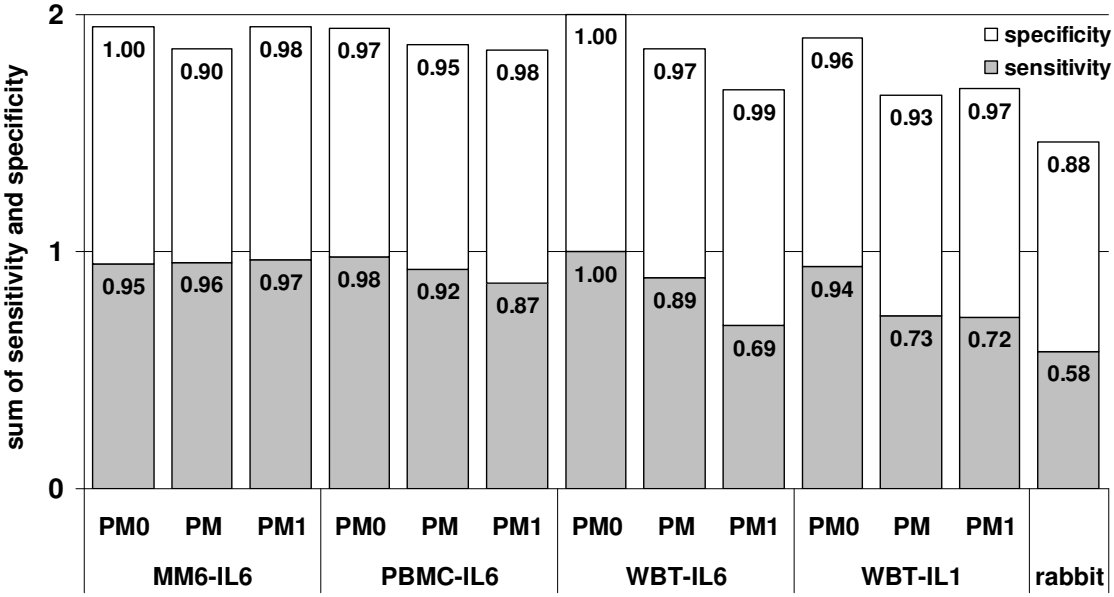

Fig. 3. Sum of sensitivity and specificity resulting from three prediction models for four in vitro assays in the validation study

The validation data of four tests were analysed with three prediction model 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 specificity and sensitivity allowing also for individual parameter assessment. For comparison, the rabbit test performance according to the pre-defined prediction model is added. The DLs also tested lipoteichoic acid (LTA) from *Bacillus subtilis*, a BET-negative Gram-positive compound that activates cytokine release from human monocytes (26, 57) prepared according to Morath et al. (57), which was clearly detectable by the novel tests.

4.5. Discussion

Previous work (49, 65, 67, 70, 136, 143, 147, 150, 151) had established that different sources of human monocytoid cells are valuable tools for mimicing 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 (58), exotoxins (67, 148), cell wall components like muramyl dipeptide (148) or peptidoglycan (149), *S. aureus* Cowan (SAC) (147) or DNA (67) as well as poly (I:C) (147), 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 (64, 67, 147, 148,). For the first time, six of these monocytoid-cell based in vitro pyrogen tests were formally validated in the present study. 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 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 the use with cells and caused their 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 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 on 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 put main emphasis on the threshold of 0.5 EU/ml. Similarly, decreased sensitivity when given the task of identifying 1 EU/ml as 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 underlined.

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

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'.

4.6. Acknowledgements

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5 Cryopreservation of human whole blood for pyrogenicity testing

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Abbreviations:
DMSO, dimethylsulfoxide; ELC, endotoxin limit concentration; EU, endotoxin equivalent units; IL-1β, interleukin-1β; LPS, lipopolysaccharide, endotoxin; LTA, lipoteichoic acid; MVD, maximal valid dilution; RT, room temperature; WHO, World Health Organization

5.1. Abstract

Human whole blood assays are increasingly employed to test immune functions or detect pyrogenic contaminations, since they offer advantages such as easy performance, few preparation artifacts and physiological cell environment. The approach, however, is often limited by availability of freshly drawn blood, putative safety concerns in case of infected donors and interindividual donor differences. To overcome these limitations, a method was developed and optimized to produce batches of cryopreserved blood that can be used directly after thawing without any washing steps. Mononuclear cells remained intact as

shown by FACS analysis. Cytokine release could be induced by a variety of immunological stimuli. The cell preparation released higher amounts of IL-1β and IL-6 compared to fresh blood, which could be attributed to the presence of the cryoprotectant DMSO. Large batches of cryopreserved blood could be produced by mixing blood donations of up to ten donors, independent of differing blood groups. The detection limit for the WHO LPS reference preparation (EC-6) with regard to induction of IL-1β release was at least 0.5 EU/ml. Endotoxin spikes at the limit concentrations prescribed by European Pharmacopoeia could be detected in a series of drugs, showing that the In vitro Pyrogen Test (IPT) can also be run with cryopreserved blood. Further possible applications include high-throughput screening for immunomodulators or toxins as well as preservation of patient samples for later analysis of cell functions.

Key words: Cryopreservation, Blood, Endotoxin, Interleukin-1β, In vitro Pyrogen Test (IPT)

5.2. Introduction

Cryopreservation of cells represents a standard procedure in cell culture. Human primary leukocytes are cryopreserved on a routine basis, for example to store human bone marrow cells (151). Further cryopreservation protocols have been established for various isolated blood cell populations including lymphocytes and mononuclear cells and the retention of various cell functions after thawing has been investigated (72, 152-155).

Although it is popular to isolate the respective immune cells from blood, it is evident that such isolated cells do not reflect the in vivo situation: the cells are often stimulated during the isolation procedure as indicated by basal mediator release or adherence of the cells, interaction between different cell types cannot take place and plasma components that often play an important role in immune recognition are no longer present.

Methods employing whole blood have been developed to detect pyrogenic (fever-inducing) contaminations, e.g. of batches of injectable drugs (49). This

application has been successfully validated in a collaborative European study and awaits incorporation into the pharmacopoeias (71). Further, we have suggested the study of cytokine, histamine or eicosanoid release with this method to allow the characterization of putative drugs or immunotoxins (156, 157). These methods can also be used ex vivo on treated volunteers or patients to monitor the course and effects of treatment (158-160).

Many of these procedures could be simplified or optimized by the availability of cryopreserved whole blood. The blood could be supplied in the form of a standardized test reagent which could be stored until needed and be certified free of infectious agents. A method to preserve and store cells from treated patients might allow performance of the often laborious cell assays on a series of collected samples in parallel or at a distant laboratory, thus reducing variability and logistical problems.

We sought to develop a protocol which would allow the use of the thawed whole blood samples directly without any washing steps to remove the cryoprotectant, as such a step would eliminate essential advantages of the human whole blood assay, i.e. the ease of performance which allows a high degree of standardization as shown for various applications (161). Furthermore, beside stress and handling artifacts, the cells would lose their autologous plasma that enables a number of physiological responses, e.g. the sensitive response to lipopolysaccharides (endotoxin, LPS) via lipopolysaccharide binding protein (LBP) (162, 163).

In this report we describe the development of a protocol to freeze human whole blood and demonstrate retention of sensitivity and functionality regarding stimulation of cytokine release in response to inflammatory agents.

5.3. Materials and methods

Freezing procedure

Blood was drawn from healthy volunteers into tubes containing 15 IU/ml Li-Heparin (Sarstedt, Nürnbrecht, Germany) and differential blood cell counts were performed on each sample to rule out active infections (Pentra 60, ABX

Diagnostics, Montpellier, France). In order to rule out pyrogenic contaminations of any component used in the incubations, negative saline controls were included in each experiment The heparinized blood was pre-cooled in ice water for 15 minutes. Clinical grade dimethylsulfoxide (DMSO, Waco Chemicals, Dessau-Thornau, Germany) was added to the blood in 50 ml centrifugation tubes (Greiner bio-one, Frickenhausen, Germany) in small amounts to a final concentration of 10% (vol/vol ratio) under constant gentle agitation to avoid cell damage. Pooling was performed in 50 ml centrifugation tubes after the addition of DMSO to the blood of the individual donors. Blood was pipetted as 1, 3 or 4 ml aliquots into pre-cooled cryotubes (1.8, 3.6 or 4.5 ml, Nunc, Wiesbaden, Germany) and put into the rack of a programmable freezer with a TP type nitrogen container (Nicool Plus PC, Air Liquide, Marne-la-Vallée Cedex 3, France), pre-cooled to 4°C. A temperature probe was inserted into an extra aliquot containing the same volume of blood to follow the freezing process. The freezing program was started 5 min after closing the freezer. The blood was cooled down to – 5°C at a rate of 1°C/minute. In order to compensate the latent fusion heat generated by the blood when changing from the liquid to the solid state, the temperature Tx in the freezing chamber was set to – 30°C. The crystallization temperature was –12°C. When this temperature was reached, the blood was cooled down to – 40°C at a rate of 2°C/min. The blood was given 120 seconds to stabilize before being cooled down to –120°C at a rate of 10°C/minute. After freezing, the tubes were removed from the freezer and put immediately into the vapor phase of liquid nitrogen (nitrogen tank, Air Liquide, Kryotechnik, Düsseldorf, Germany).

Thawing procedure

The closed tubes were left in an incubator at 37°C until completely thawed.
The aliquots of single donors were either pooled or the blood was pipetted individually from each aliquot. Pooling of the blood of different donors could be performed after thawing as an alternative to the procedure described above. The whole blood incubation was started not more than 30 minutes after complete thawing.

Whole blood incubation

Human whole blood incubations were performed according to the protocol of the in vitro Pyrogen Test (IPT; 49, 71). Briefly, 100 µl of fresh or cryopreserved human blood were added to 1 ml physiological, clinical grade, pyrogen-free saline in polypropylene reaction tubes (Eppendorf, Hamburg, Germany). After the addition of stimuli, the tubes were closed, shaken gently and incubated overnight (16-24 hours) at 37°C. The cells were resuspended and assayed immediately or frozen until measurement. Within each experiment performed, all samples were incubated and measured in parallel. When all samples of an experiment were measured on the same ELISA plate, absorbance (A 450) was given as the unit of measurement according to the IPT protocol. When the samples could not be measured on the same ELISA plate, a recombinant standard curve was run on each ELISA plate to allow interpolate comparison.

Endotoxin stimuli were LPS from *Escherichia coli* O113 (WHO standard material), kindly provided by Dr. Stephen Poole, NIBSC, Hertfordshire, GB, or LPS from *E. coli* O111 (IPT Kit, Charles River Endosafe, Charleston, SC, USA) calibrated to the WHO standard material. One important criterion for the In Vitro Pyrogen Test (IPT) is the ability to reproducibly detect the presence of 0.5 EU (endotoxin equivalent units) per ml, equivalent to 50 pg/ml of the WHO reference endotoxin standard or to 100 pg/ml of the LPS from *E. coli* O111, respectively, in a sample solution, this being the fever threshold of the most sensitive rabbit strain if applied at a dose of 10 ml/kg. Therefore, this LPS concentration was included in every assay.

Non-endotoxin stimuli were lipoteichoic acid (LTA) from *Bacillus subtilis* (IPT Kit, Charles River Endosafe) (49), glucan standard (Charles River Endosafe), glucan from barley (Sigma, Munich, Germany), lectin from *Phaseolus vulgaris* (PHA-L and PHA-E, Sigma), curdlan (Sigma) and zymosan A (Fluka, Buchs, Switzerland).

Substances tested at MVD were furosemid (Lasix®), ampicillin (Binotal®), Articain/Epinephrin (Ultracain®) (Aventis, Germany), Theophyllin (Bronchoparat®), (Fujisawa, Munich, Germany), dimethindenmaleat (Fenistil®) (Novartis, Munich, Germany), ranitidin (Sostril®) (Glaxo Smith

Kline, Munich, Germany) and metoprolotartrat (Beloc®) (Astra Zeneca, Wedel, Germany).

Cytokine ELISAs were based on commercially available antibody pairs against IL-1β or TNFα (Endogen, Biozol, Eching, Germany), and IL-6 (R&D, Wiesbaden, Germany). Binding of biotinylated antibody was quantified using streptavidin-peroxidase (Biosource, Camarillo, CA, USA) and the substrate TMB (3,3',5,5'-tetramethylbenzidine, Sigma). Recombinant cytokines serving as standards were gifts from Dr. S. Poole, NIBSC.

FACS Analysis

25 µl of fresh or cryopreserved blood was stained with 5 µl each of anti-CD45-APC and anti-CD14-FITC antibodies (BD Biosciences, Heidelberg, Germany) for 30 min at room temperature in the dark. 1 ml Cell Wash and propidium iodide in a final concentration of 500ng/ml were added directly, immediately before measurement in a FACSCalibur (all BD Biosciences). A live gate was set on CD45-positive cells and 3000 leukocytes were counted. Whole blood counts were determined by Türks staining and counting in a Neubauer chamber.

Statistics

Statistics were performed with GraphPad InStat 3.0 (GraphPad Software, San Diego, USA). Significance was tested by one-way ANOVA and Dunnetts post-test/Dunn´s multiple comparison and with t-test, followed by Mann-Whitney post-test.

5.4. Results

Freezing procedure

Different concentrations of the cryoprotectant DMSO were tested to determine a concentration that would protect the cells and leave them functional after thawing but which would be sufficiently low to have no toxic effects in the

incubation (Fig. 1). While 1% DMSO was insufficient to protect the cells during freezing and 20% DMSO had toxic effects in the subsequent incubation period, cell preparations frozen with either 5 or 10% DMSO responded to stimulation with LPS in a concentration-dependent manner after thawing.

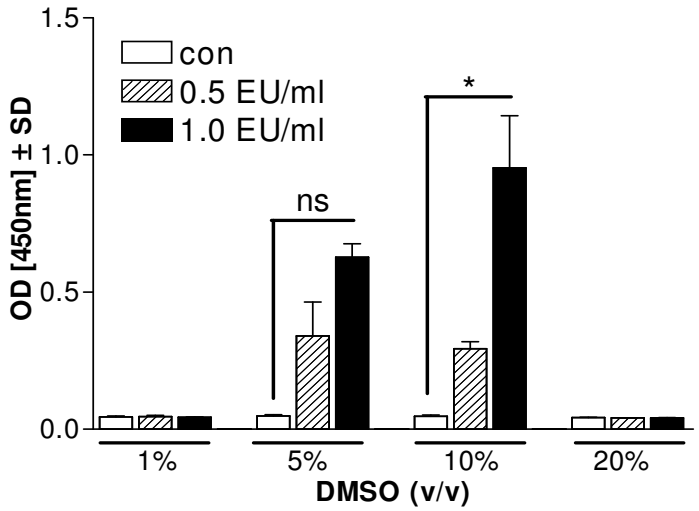

Fig. 1. Optimization of the final DMSO concentration in cryopreserved blood.

Blood was frozen with different concentrations of DMSO as shown and stimulated with LPS from *E. coli* O113 after thawing (representative experiment of 2). Blood from one donor in 4 replicates is shown, *, $p<0.05$ (one-way-ANOVA, post test: Dunn´s multiple comparison). Incubation supernatants were measured by ELISA technique and the endpoint IL-1β was
given as OD.

We compared whether the reactivity of the cryopreserved blood measured as IL-1β response to endotoxin stimulation was affected by the blood temperature (room temperature or 4°C) at which the DMSO was added and whether DMSO should be added as a bolus or in several aliquots. The addition of DMSO at room temperature seemed to cause an increase in reactivity rather than a decline and addition of DMSO in several aliquots was preferable to the bolus.

The mean OD of the aliquots with DMSO added at room temperature and stimulated with 0.5 EU/ml O113 was 0.596 OD (SD 0.049) when the DMSO was added as a bolus and 0.728 OD (SD 0.051) when the DMSO was added in three aliquots. The cv was 8.17 and 7.02%, respectively. When the DMSO was added at 4°C, the response was 0.404 OD (SD 0.056) when the DMSO was added as a bolus and 0.547 (SD 0.034) when added in three aliquots (cv 13.82 and 6.29%).

Next, we determined how long the blood could be kept after addition of DMSO before freezing and whether room temperature or 4°C is preferable. For this purpose, DMSO was added to the blood and an aliquot was frozen immediately while other aliquots were stored at room temperature or at 4°C for up to 200 minutes and then frozen and tested in parallel (Fig. 2). These data suggest that storage at room temperature for up to 2 hours is tolerable and that storage at 4°C is beneficial when the blood is stored for longer.

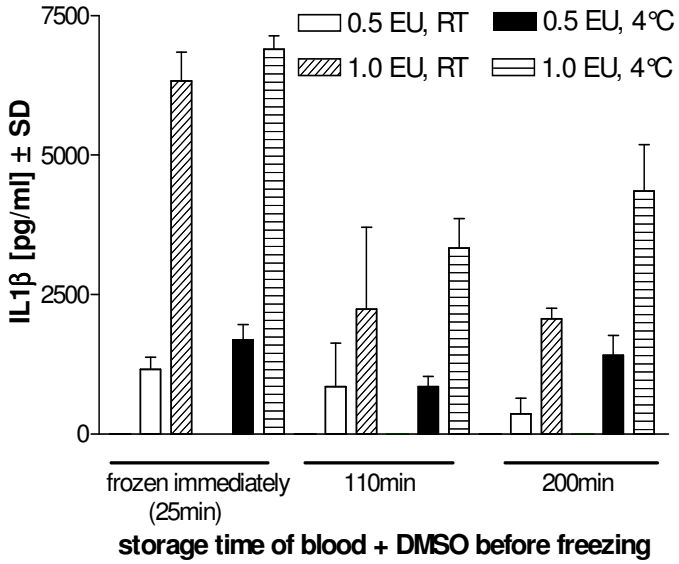

Fig. 2: Comparison of different storage temperatures and durations before freezing.

Blood with 10% DMSO was frozen immediately or stored as indicated before freezing, then stimulated with LPS from *E. coli* O111 after thawing (representative experiment of two). Control values were < 6 pg/ml IL-1β for

each condition. Four replicates of blood from one donor are shown (0.5 EU/ml: n=5).

Comparison of different volumes

Different volumes of blood (1 ml, 3 ml and 4 ml aliquots) were frozen and stimulated with endotoxin after thawing. The reactivity of the blood did not depend on the volume of the frozen aliquots. The mean OD of the 1, 3, and 4 ml aliquots when stimulated with 1.0 EU/ml of O113 was 2.824 (SD 0.066), 2.463 (0.058) and 2.6 (0.087) OD, respectively, with a coefficient of variation of 2.32 , 2.35 and 3.35%.

Thawing procedure

A thawing protocol was developed in order to optimize the handling of the blood aiming at maximum reactivity and viability. Aliquots of blood from the same donors were thawed under different conditions, i.e. on ice, at room temperature (20°C) and in an incubator (37°C) until completely thawed before stimulation with endotoxin (Fig. 3). Quick thawing at 37°C resulted in the best response.

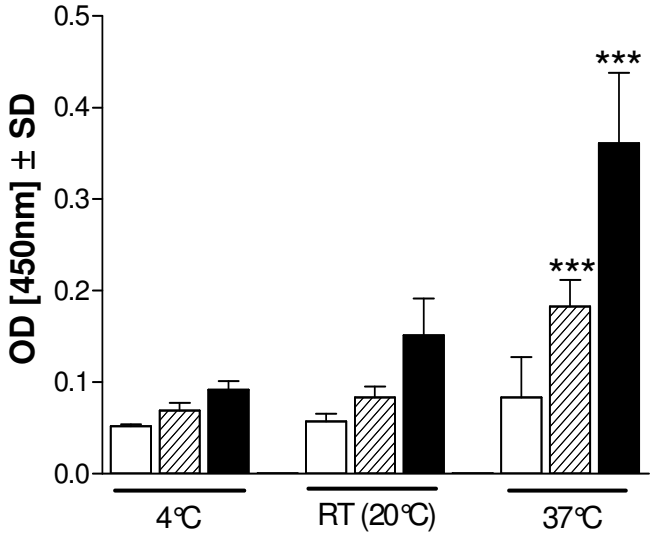

Fig. 3: Determination of a suitable thawing temperature.

Cryopreserved blood with 10% DMSO was thawed at different temperatures (4°C, room temperature, 37°C), then stimulated with 0.5 EU/ml (hatched bars) and 1.0 EU/ml (black bars) LPS from *E. coli* O113 or saline (white bars) (representative experiment of 4). Blood from one donor in 8 replicates is shown. ***, $p<0.001$ against the respective stimulation after thawing at 4°C or 20°C (one-way ANOVA with Dunnett's post-test). Incubation supernatants were measured by ELISA technique and the endpoint IL-1β was given as OD.

An important issue was the potential cytotoxicity of the remaining cryoprotectant DMSO after thawing and before dilution with saline. Therefore, we tested how long the thawed blood samples could be kept at 37°C before dilution and stimulation (Fig. 4). The reactivity of the blood towards the endotoxin stimulation decreased after 45 minutes of thawing time. Therefore, the blood was used within 30 minutes after thawing at 37°C in all subsequent experiments.

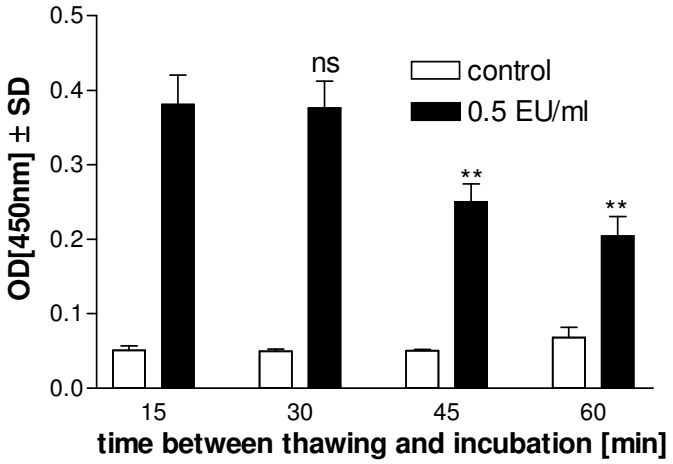

Fig. 4: Effect of time between thawing of blood and incubation.
Blood was thawed and stored at 37°C for the times indicated, then stimulated with 0.5 EU/ml LPS from *E. coli* O113 (representative experiment of 4). Blood from one donor in five replicates is shown. ** = $p<0.01$ vs. the values at 15 min

(one-way ANOVA with Dunnetts post test). Incubation supernatants were measured by ELISA technique and the endpoint IL-1β was given as OD.

FACS Analysis

Differential blood cell counts were done in parallel samples of fresh and cryopreserved blood from 5 donors. Although the whole blood cell counts of the cryopreserved blood did not differ from those of the fresh blood samples,
the differential blood cell count revealed that the neutrophilic granulocytes had lost their surface markers and could no longer be identified as live, CD45 positive cells. The ratio of monocytes to lymphocytes in the differential blood cell count was the same in the fresh and the cryopreserved blood (1 : 6.7 ± 0.9 versus 1 : 8.1 ± 1.6, n.s.) with a viability of these two populations of 99 vs. 90% as shown by propidium iodide exclusion.

Establishment of a pooling protocol

Blood samples from five different donors were compared with each other and with pools of the blood from the same donors combined either directly after addition of the DMSO or after thawing of frozen blood. Establishing a pooling protocol with blood from different donors with different blood groups proved easier than anticipated (Fig. 5). There was no difference in the reactivity of the blood pools, whether they were made before or after freezing. Also, the reaction of the pooled blood was equal to the mean of the reaction of the individual donors.

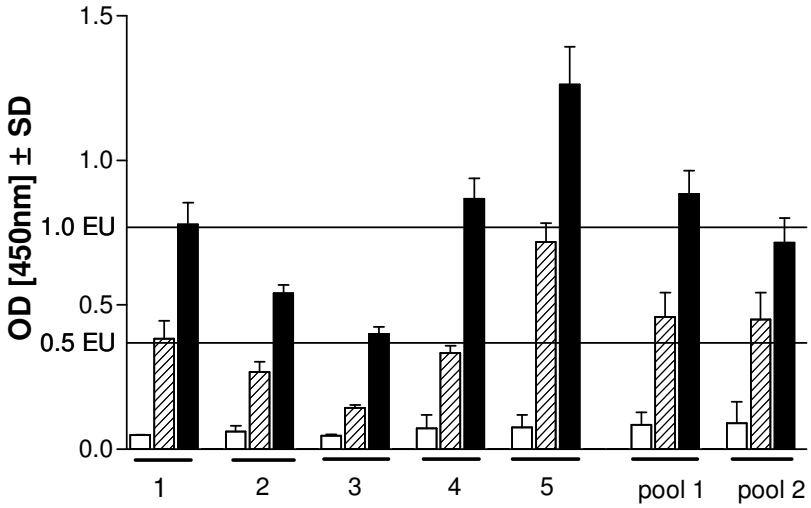

Fig. 5: Comparison of the reactivity of frozen blood from 5 donors and their pooled blood.

Blood from five separate donors as well as a pool of their blood was frozen and stimulated as shown (representative experiment of 4). The horizontal lines indicate the calculated mean of the blood from the five donors to 0.5 or 1.0 EU/ml LPS from *E. coli* O113. Three replicates of all samples were measured (0.5 EU/ml: 4 replicates). Pool 1, the blood of the single donors was pooled after addition of DMSO; pool 2, the blood was pooled after thawing. Incubation supernatants were measured by ELISA technique and
the endpoint IL-1β was given as OD.

Interlot variability

5 different pools of cryopreserved blood, each containing the blood of 5 donors, were compared (Fig. 6). The interlot variability was very low, indicating that the use of 5 donors in the pooling protocol is sufficient for producing highly similar batches of blood.

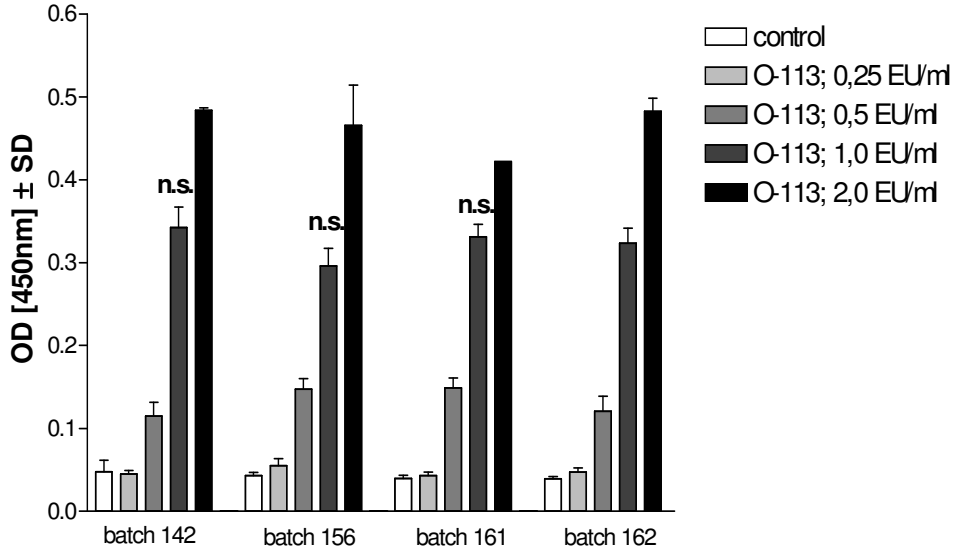

Fig. 6: Interlot variability of five different pools

Cryopreserved pools each consisting of five different donors and frozen over a period of 23 weeks were thawed and stimulated on the same day (0.25 EU/ml, 2 EU/ml: 2 replicates each; control, 0.5 EU/ml, 1 EU/ml: 4 replicates each) with LPS from *E. coli* O113. $p > 0.05$ of the 1.0 EU/ml value of batch 142-161 against the 1.0 EU/ml value batch 162 (one-way ANOVA, Dunnett´s post-test). Incubation supernatants were measured by ELISA technique and the endpoint IL-1β was given as OD.

Stability

Numerous aliquots of a pool of blood from 5 donors were frozen and their reactivity tested on different days over a period of 4 months. The IL-1β response to 0.5 EU/ml endotoxin was significantly different from the saline controls at each of the time points tested, indicating that the cryopreserved blood remained stable over this time period and did not lose sensitivity (Table I).

Day after freezing	Mean OD saline control	Mean OD 0.5 EU/ml	Reactivity (% saline control)
0	0.045 ± 0	0.311 ± 0.06	691
40	0.068 ± 0.01	0.298 ± 0.02	439
118	0.077 ± 0.01	0.755 ± 0.05	980

Table I: Stability of pooled cryopreserved blood from 5 donors over a period of four months.

Blood was stimulated with 0.5 EU/ml LPS from *E. coli* O113, n=4. Incubation supernatants were measured by ELISA technique and the endpoint IL-1β was given as OD.

To determine inter-aliquot variability of aliquots from the same blood donor, eight replicates each from 3 aliquots of 1 ml thawed cryopreserved blood were stimulated with 0.5 EU/ml endotoxin and eight replicates each from three aliquots were left unstimulated (Table II). The stimulated samples had mean ODs of 0.27 – 0.49 and the coefficient of variation (cv) was 12.3 – 26.1%, while the unstimulated samples had mean values ranging from 0.047 to 0.054 OD and a cv of 5.4 – 42.2%.

The inter-aliquot variability of the same experiment was 0.051 ± 0.004 (cv 7.3%) for unstimulated versus 0.37 ± 0.11 (cv 31.3%) for blood stimulated with 0.5 EU/ml LPS.

	aliquot 1 saline	aliquot 2 saline	aliquot 3 saline	aliquot 4 0.5 EU/ml	aliquot 5 0.5 EU/ml	aliquot 6 0.5 EU/ml
Minimum	0.040	0.043	0.048	0.242	0.216	0.384
Median	0.046	0.047	0.053	0.259	0.317	0.495
Maximum	0.107	0.051	0.066	0.339	0.490	0.582
Mean	0.053	0.047	0.054	0.274	0.327	0.493
SD	0.022	0.003	0.006	0.036	0.085	0.061

SEM	0.008	0.001	0.002	0.012	0.030	0.021
cv (%)	42.17	5.4	10.66	12.83	26.08	12.26

Table II: Intra-aliquot variability of cryopreserved blood from one donor. 8 replicates from each aliquot of blood of the same donor were stimulated with LPS from *E. coli* O113. Incubation supernatants were measured by ELISA technique and the endpoint IL-1β was given as OD.

Comparison of the reactivity of cryopreserved with fresh whole blood

The reactivity of the cryopreserved blood to endotoxin stimulation was compared to that of fresh blood of the same individual donors. As can be seen in Figure 7, 0.5 EU/ml LPS induced significant IL-1β release both in the fresh and the cryopreserved blood of every donor. This is the sensitivity limit of the most sensitive rabbit strain for testing according to the European Pharmacopoeia for injectable drugs.

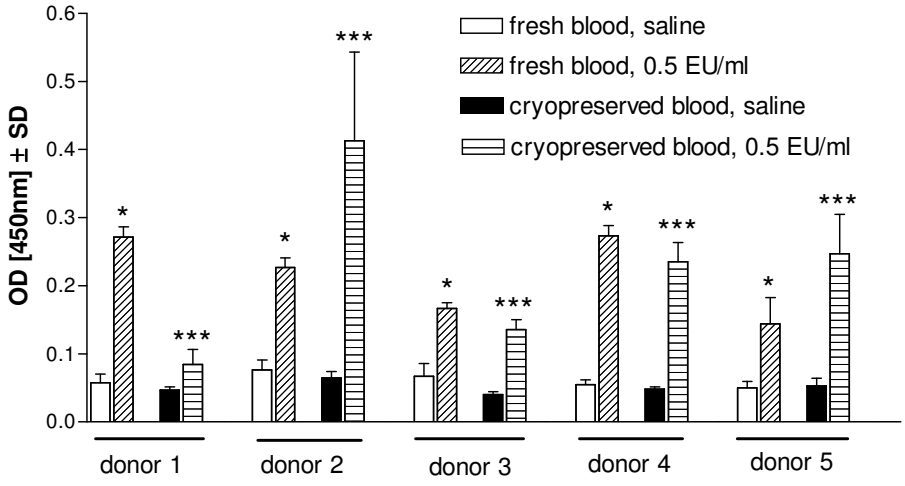

Fig. 7: Comparison of the reactivity of fresh and frozen blood of 5 separate donors.

Fresh blood (4 replicates each) and thawed cryopreserved blood (6 replicates each, saline control n=8) from the same five donors was stimulated with LPS from *E. coli* O113. Cryopreserved blood was thawed immediately after complete freezing and was incubated in parallel with the fresh blood of the same donors (representative experiment of 3).

*, $p<0.05$, *** $p<0.001$, against the respective saline control (t-test and Mann-Whitney post-test). Incubation supernatants were measured by ELISA technique and the endpoint IL-1β was given as OD.

When the response to endotoxin stimulation of cryopreserved and fresh blood from the same donors was compared in a kinetic study, a noticeable difference in the kinetics of the LPS-inducible IL-1β release was observed (Figure 8, upper panel). Measurable IL-1β release occurred with several hours delay in cryopreserved compared to fresh blood. This could be attributed to the presence of the cryoprotectant, since fresh blood containing 10% DMSO showed the same delay. Furthermore, in both cases, the presence of DMSO increased the maximum amount of IL-1β released 7-fold (fresh blood plus DMSO) and 5-fold (cryopreserved blood). The same held true for IL-6 (Fig. 8, center panel), though here the amount of IL-6 was increased nearly 20-fold. TNFα release was no longer detectable after addition of DMSO (Fig. 8, lower panel), both in fresh and cryopreserved blood.

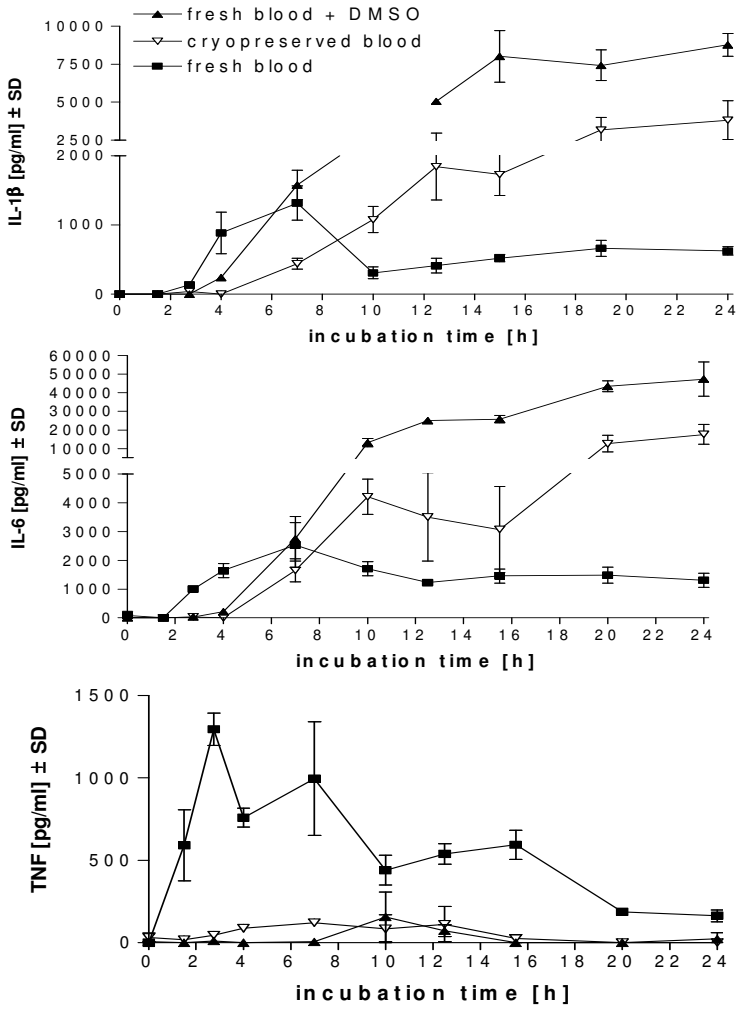

Fig. 8: Kinetics of cytokine response of fresh blood, fresh blood after addition of 10% DMSO and cryopreserved blood.

Three replicates of blood samples pooled from five donors were challenged with 1.0 EU/ml LPS from *E. coli* O111 for the times indicated.

Next, the reactivity of cryopreserved blood to a variety of immune stimuli was tested in comparison to fresh blood. Different pyrogenic (fever-inducing) stimuli including LPS, LTA, and phytohaemagglutinin-L (PHA-L) induced IL-1β release in cryopreserved blood, but not the non-pyrogenic substances PHA-E, glucans and monophosphoryl lipid A (data not shown). Differences were seen for curdlan and zymosan A and, in terms of a higher sensitivity of the cryopreserved blood, for endotoxin from *Pseudomonas aeruginosa*. Taken