Novel Pyrogen Tests Based on the Human Fever Reaction

The Report and Recommendations of ECVAM Workshop 431,2

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Preface

This is the report of the forty-third of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM’s main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences, and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well informed about the state of the art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would best be achieved by the organisation of ECVAM workshops on...
specific topics, at which small groups of invited experts would review the current status of various types of in vitro tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on novel pyrogen tests based on the human fever reaction was held in Konstanz, Germany, on 16–20 January 2000, under the chairmanship of the local organiser, Thomas Hartung (University of Konstanz). The participants came from industry, academia and national control authorities (in Europe and the United States), and their expertise extended to pharmacology and toxicology, quality control and quality assurance, immunology, biochemistry, microbiology, laboratory animal science, human medicine, and the development of in vitro assays as alternatives to animal testing, including the pertinent regulatory procedures.

The objectives of the workshop were to identify the need for new pyrogen tests; to review the current status of the development of new pyrogen tests; to evaluate the capabilities of the new pyrogen tests and to give recommendations for further development; to identify regulatory requirements and to give recommendations for promotion to regulatory acceptance; and to summarise the EU project “Human(e) Pyrogen Test” (QLRT-1999-00811; see Appendix 1).

The outcome of the discussions and the recommendations agreed by the workshop participants as individuals are summarised in this report.

Introduction

Pyrogenicity

Fever is one of the main symptoms of infectious diseases caused by bacteria, viruses or parasites (2, 3). However, the fever reaction is not directly connected to live microorganisms. At the end of the 19th century, fever-inducing contaminations were found to be heat-stable. Soon afterwards, the connection was established between pyrogenicity and the heat-stable endotoxin originating from Gram-negative bacteria. Because the mechanisms of fever generation were unknown at that time, the fever-causing materials were classified as either exogenous or endogenous pyrogens. Exogenous pyrogens are materials from the environment (for example, debris from microorganisms) that cause fever in mammals. Until now, endotoxins, a group of chemically similar cell-wall structures of Gram-negative bacteria, are the best-characterised exogenous pyrogens, and these have been shown to be lipopolysaccharides (LPS; 4–9). LPS are the main components of the cell walls of Gram-negative bacteria. Fever also occurs on infection with Gram-positive bacteria, viruses or parasites, but little is known about the structures that cause this reaction. All these materials, however, appear to interact with peripheral blood cells (10), which subsequently release messenger substances to transmit signals to the hypothalamus, to elevate the body temperature (11–18). These humoral transmitters are called endogenous pyrogens, and their chemical identities are interleukin-1 (IL-1; 19, 20), interleukin-6 (IL-6; 21, 22), tumour necrosis factor-alpha (TNF-α; 23, 24) and prostaglandin E2 (PGE2; 25, 26).

Conventional pyrogen tests

Rabbit pyrogen test
In this report, the term “rabbit pyrogen test” refers to the animal test as defined in the pharmacopoeias. The presence of pyrogens leads to an increase in the body temperature of rabbits. Contamination with pyrogens is hazardous, especially in drugs for intravenous use. Since numerous exogenous pyrogens are heat-stable, sterilisation is not sufficient to exclude fever reactions. In the 1940s, the rabbit pyrogen test was introduced as an analytical control procedure for the detection of fever-causing contamination, and was consequently incorporated into various pharmacopoeias and guidelines.

However, the rabbit pyrogen test has several drawbacks. The sensitivity of rabbits toward endotoxin reference preparations depends on the strain used and the experimental conditions (for example, age, gender and housing conditions; 27, 28). Nevertheless, the rabbit strain to be used is not defined in the respective monographs of the pharmacopoeias. Even if the highest permitted volume (10ml/kg body weight) is injected, the detection limit is restricted to 50–350pg (i.e., 0.5–3.5IU) of LPS/kg; however, the human fever threshold is around 30pg/ml (29, 30) and, for many drugs, the volume tested is significantly smaller.

Drugs that are to be intravenously injected must be shown to be of a pyrogen-free quality. For other parenteral drugs, given subcutaneously or intramuscularly in much smaller
volumes (for example, vaccines), a maximal acceptable endotoxin concentration has to be defined for quality-control purposes. However, the rabbit pyrogen test is not suitable for the control of such a limit since it is not a quantitative test, i.e. it gives only a pass/fail result, and it is less-well standardised (31, 32).

Bacterial endotoxins test
The term “bacterial endotoxins test” (BET) refers to a number of tests that are also described in the pharmacopoeias, and which primarily detect endotoxins from Gram-negative bacteria. Their presence results in the clotting reaction of the amoebocyte lysate of horseshoe crabs. The test is historically referred to as the Limulus amoebocyte lysate (LAL) test, although lysates from both Limulus polyphemus and Tachypleus tridentatus are employed. In this report, the two terms, BET and LAL, are used synonymously.

In the 1970s, radiopharmaceutical drugs were introduced into clinical practice. These drugs cannot reasonably be tested in rabbits. The solution was the LAL test (33–35) which, over the last 30 years, has replaced the rabbit pyrogen test for these and a number of other products (36–38).

The LAL reaction is primarily based on the clotting reaction of the haemolymph of the horseshoe crab in the presence of bacterial endotoxins. There are various approaches for measuring the LPS-induced reaction (for example, clotting reaction, kinetic turbidimetric measurement, chromogenic endpoint and kinetic reaction; 39, 40), which show some dissimilarities (41, 42). This test is specific for endotoxins from Gram-negative bacteria, and precisely because of this property does not detect pyrogenic substances other than endotoxins. Nevertheless, in most cases, endotoxins represent the leading pyrogenic contamination, so it has generally been possible to assure “pyrogen-free” products with the LAL test (43). The main advantage of the LAL test is the availability of an endotoxin standard that permits the semi-quantitative or quantitative measurement of endotoxins (44–46). The detection limit is usually about 3pg/ml, i.e. 0.031U/ml (1.5pg/ml final concentration). More-sophisticated LAL systems show a higher sensitivity. However, the relative potencies of individual endotoxins from various species might differ in LAL and in mammalian assays (47, 48).

The LAL test is undoubtedly suitable for the pyrogen testing of a number of products, but not for all products. For example, certain biological drugs interfere with the LAL test, and therefore are still tested by using the rabbit pyrogen test. The LAL test is not applicable to the detection of endotoxins (or other pyrogens) adsorbed onto inorganic or organic surfaces.

Other fields in which pyrogen testing is relevant but not sufficiently applied, i.e. applications to be developed to optimise patient, consumer and workplace safety, include the testing of medical devices (49), cellular blood components and other cellular therapeutics (50, 51), species-specifically acting recombinant proteins (for example, immunostimulators), and air pollution control (52).

Conclusions from positive results in conventional pyrogen tests with regard to a fever reaction in humans
Positive reaction in the rabbit pyrogen test
A positive result in the rabbit pyrogen test demonstrates contamination with pyrogens according to the requirements and definitions of relevant monographs and guidelines. However, there remains a gap between the observed pyrogenicity in rabbits and the expected pyrogenicity in humans. Due to species differences, some materials (for example, biologicals for human use) might cause fever in humans but not in rabbits, and vice versa.

Positive reaction in the LAL test
A positive result in the LAL test is sufficient proof that the product is pyrogenic in humans. Nevertheless, a negative result does not mean that the product is pyrogen-free, since non-endotoxin pyrogens are not detected with the LAL test.

Fever reaction in humans
A fever reaction in humans, which is clearly associated with a sample or material, demonstrates pyrogenicity. However, such samples are rarely available, the documentation of the event in the clinic is often not available, and a change over time as well as in an individual’s condition (idosyncratic reaction of the patient) cannot be excluded. Whenever possible, such samples should be included in evaluation and validation efforts.

The Need for Novel Pyrogen Tests
In this report, the term “novel pyrogen test” refers to systems that can detect endotoxins
and other pyrogens in vitro by measuring the release of fever-inducing mediators.

The advantages and limitations of the rabbit pyrogen test and the LAL test have already been discussed in detail in the Introduction, and are briefly summarised here. The advantage of the rabbit pyrogen test is that it can detect endotoxins and non-endotoxin pyrogens. It is limited by a number of factors: it is not quantitative; it is less sensitive than the LAL test; the outcome depends on the rabbit strain and housing conditions; it is not suitable for all product categories; it is expensive; and it involves the use of animals.

The most important difference between the rabbit pyrogen test and the LAL test is the failure of the latter to detect non-endotoxin pyrogens. On the other hand, the BET makes it possible to test the pyrogenicity of products that have not been found to be testable in the rabbit, for example, radiopharmaceuticals. The recently established, more sophisticated, kinetic and colorimetric BET systems have increased sensitivity. Nevertheless, there remain a huge number of complex preparations, such as biologicals, which cannot be tested with BET systems.

Therefore, initially, a novel pyrogen test should combine the advantages of the rabbit pyrogen test and the BET, without suffering from their disadvantages. It should be robust, quantitative, sensitive, applicable to a wide variety of products, and it should detect non-endotoxin pyrogens.

The limitations of the rabbit pyrogen test and the BET systems demand the establishment of advanced pyrogen test methods, which should meet the following specifications: a) they should not involve animals; b) they should mimic the human fever reaction; c) they should detect a broad range of pyrogens; d) they should be widely applicable (minimal interference with the products to be tested); e) they should be quantitative or semi-quantitative; and f) they should be simple and practicable (the reagents should be available in less-well-equipped laboratories).

Current Status of Novel Pyrogen Tests

In recent years, a number of new approaches to pyrogen testing have been reported, which are mainly based on the use of human cells. Pyrogens induce the release of mediators that can be quantified by immunochemical methods. The three main approaches involve the use of cells of various origin: leucocyte cell lines, isolated primary blood and human whole blood. Some of the workshop participants have been involved in the development of such in vitro pyrogen tests, which are briefly outlined in the following paragraphs. Their key characteristics in comparison with the rabbit pyrogen test and bacterial endotoxin tests are summarised in Table I.

<table>
<thead>
<tr>
<th>Novel pyrogen tests based on leucocyte cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Principle</strong></td>
</tr>
<tr>
<td>Human monocyte/macrophage-like cell lines, such as MonoMac-6 and THP-1, or clones derived from such lines and murine macrophage-like cells, such as RAW264.7, have been widely used for pyrogen testing. Pyrogens induce cytokine production (IL-6, TNF-α) or lead to the formation of metabolites (neopterin, nitrite) from cytokine-inducible pathways cells, which can then be measured in the supernatants of the cultured cells by ELISA methods (IL-6, TNF-α, neopterin) or detected spectrophotometrically through the Griess reaction.</td>
</tr>
<tr>
<td><strong>Examples</strong></td>
</tr>
<tr>
<td>MonoMac-6. MonoMac-6 cells (53) are incubated overnight in Iscove's modified Dulbecco's medium (IMDM) supplemented with Glutamax-1, 2% fetal calf serum (FCS) and 100U penicillin/streptomycin, in 96-well, flat-bottomed, tissue culture plates. Commercially available ELISA kits are used for detection of IL-6 or TNF-α. The test system has been compared with the rabbit pyrogen test (specific pathogen-free rabbits) and the LAL test (gel clot); the sensitivity was 0.125IU/ml for Endotoxin Standard Biological Reference Preparation Batch No. 2 (European Pharmacopoeia [Ph. Eur.]) compared with 0.03IU/ml for LAL. The limit of detection for LPS is 10pg/ml in the culture medium, and the absolute detection limit is 100pg/ml LPS. The test system shows a 100% LPS spike recovery rate in albumin. The presence of immunoglobulin G inhibits IL-6 and TNF-α detection (dilutions should be tested). For some bacterial vaccines, as shown for typhoid, pneumococcal and meningococcal polysaccharides in a pilot evaluation, the LAL test can be combined with the MonoMac-6 test.</td>
</tr>
</tbody>
</table>
| THP-1 and RAW264.7. THP-1 or RAW264.7 cells (54–58) are incubated overnight in 96-
Table I: Characterisation and comparison of various pyrogen tests

<table>
<thead>
<tr>
<th></th>
<th>Limit of detection (IU/ml)</th>
<th>Quantification</th>
<th>Accuracy</th>
<th>Precision</th>
<th>Robustness</th>
<th>Sensitivity/specificity</th>
<th>Costs</th>
<th>Acceptance</th>
<th>Spectrum</th>
<th>Practicability</th>
<th>Standardisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit pyrogen tests</td>
<td>± 0.5-3</td>
<td>-</td>
<td>n.a.</td>
<td>n.a.</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bacterial endotoxin tests</td>
<td>+ 0.03</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>±</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>In vitro pyrogen tests</td>
<td>+ 0.03-0.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>u.s.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>u.s.</td>
<td></td>
</tr>
</tbody>
</table>

*Overall judgement: + = positive, - = disadvantage, ± = intermediate*

n.a. = not applicable, *=public versus scientific opinion, b=LPS only, ^=predominantly artificial contamination, u.s. = under evaluation, IU = 100 μg
well plates, in serum-free culture medium (Ultraculture for THP-1, X-Vivo for RAW264.7, both from BioWhittaker [Verviers, Belgium]; 56–58). In order to increase their sensitivity, the cells are co-stimulated with 250U/ml of human and 50U/ml of pyrogen-free murine recombinant Interferon-γ, respectively (59). For THP-1 cells, the induction of GTP cyclohydrolase-I is determined by measuring neopterin by ELISA, whereas for RAW264.7 cells, the induction of NO synthase is determined by measuring nitrite derived from NO by reaction with molecular oxygen (the Griess reaction, OD at 540nm). Comparison with other test systems indicates that, for pyrogens from Gram-negative bacteria, the test systems correlate well with LAL test results, whereas they are superior for detection of pyrogens from Gram-positive bacteria. Correlation with results from the rabbit test and another cell-culture-based test (detection of TNF-α formed by THP-1 1G3 cells [54]) could be demonstrated for a limited number of samples (58); however, further, more-detailed, studies are needed. The limit of detection for LPS is 1–10pg/ml (depending on the LPS preparation), and pyrogens from both Gram-negative and Gram-positive bacteria are detected.

Subclones derived from the human monocytoid THP-1 and MonoMac-6 cells. Subclones derived from the human monocytoid THP-1 and MonoMac-6 cells have been selected on the basis of their high sensitivity to endotoxin, and were shown to be phenotypically stable (54, 55). The cells are primed with calcitriol for 44 hours, followed by a 4-hour exposure to the test sample. Cell culture supernatants are collected and TNF-α is determined by ELISA. Comparison with the LAL test shows a good correlation, and the tests are of comparable sensitivity; however, the cell culture method appears to be more specific. The limit of detection for LPS was 15pg/ml, i.e. 0.15IU/ml, for LPS from Escherichia coli O55:B5. Robustness and day-to-day variability are under further evaluation.

Tests based on isolated primary blood leucocytes

Monocytes and lymphocytes are isolated from blood samples by density-gradient centrifugation (for example, with Ficoll), then the washed cells are incubated in FCS-containing medium and exposed to the test sample for several hours (60–66). The production of IL-1, TNF-α or IL-6 is then measured by immunochemical methods (for example, an ELISA or a bioassay).

Data on comparison with other test systems are not available. The limit of detection is about 10pg/ml for LPS. The test systems are considered to be laborious and variable, due to the various stages in cell preparation. Nowadays, better-standardised and more-easily handled test systems based on cell lines or whole blood are available, and most of the tests involving isolated primary blood leucocytes have not been further developed or standardised. However, some such test systems are still in use, especially in industry.

Tests based on human whole blood

Principle

Fresh human whole blood is diluted and incubated in the presence of the sample to be tested. The production of IL-1β, IL-6, TNF-α or prostaglandin E2 (PGE2), as measured by using immunochemical methods, correlates with the content of pyrogens in the sample (30, 48, 67–75). The blood can be used for up to 8 hours after withdrawal, and the use of cryopreserved blood is also possible.

Examples

Human whole-blood model (endpoint IL-1β). Human whole blood (10% v/v in saline) and the test sample are incubated overnight, and IL-1 is subsequently measured in the supernatant by ELISA. A simplified one-plate assay is under development. Comparison with other systems reveals a good correlation with the rabbit pyrogen test, whereas differences are evident between the potencies of LPS derived from different bacterial species as estimated by the LAL test and the human whole-blood model. The limit of detection is <50pg LPS/ml sample (4.2pg/ml final concentration). The donor-dependent variation in the threshold of IL-1β release is low. The test system is not inhibited by up to 10% dimethyl sulphoxide; however, cryopreserved blood gives a much stronger IL-1β response to endotoxin.

Human whole-blood model (endpoint IL-6). Human whole blood (10–20% v/v in saline) and the test sample are incubated for 4 hours, and IL-6 release is measured by ELISA. A rapid one-plate pyrogen test has been developed, which combines the incubation phase with the evaluation phase in a 96-well plate, which is coated with affinity-purified monoclonal anti-
bodies against IL-6. This one-step incubation and measurement of IL-6 increases the sensitivity; thus, the limit of detection is 0.03 IU/ml, i.e. 3 pg/ml LPS, for the one-plate assay, and 0.06 IU/ml, i.e. 6 pg/ml LPS, for the two-plate assay.

Problems Associated With Pyrogen Testing

In the light of the need for better pyrogen tests, a series of questions arise with regard to problematic products that cannot be tested with the LAL or the rabbit pyrogen test, the relevance and evidence of non-endotoxins, and potential future areas for pyrogenicity testing.

Relevance and evidence of non-endotoxin pyrogens

There are a number of substances other than endotoxins that can potentially cause fever in humans and animals (76). The list of substances given in Table II is based on clinical experience, animal experiments and the induction of fever mediators in immune cells. However, in contrast to endotoxins, they have been less-well investigated, i.e. in general, a conclusive dose–response relationship

Table II: Pyrogenic non-endotoxin substances

<table>
<thead>
<tr>
<th>Substance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin associated proteins</td>
<td>77</td>
</tr>
<tr>
<td>Peptidoglycans (components of bacterial cell wall)</td>
<td>78, 79</td>
</tr>
<tr>
<td>Muramylpeptides (MDP and other subunits of peptidoglycan synergise with endotoxins)</td>
<td>80-82</td>
</tr>
<tr>
<td>Porins (proteins from the bacterial cell wall)</td>
<td>83, 84</td>
</tr>
<tr>
<td>Bacterial outer surface proteins</td>
<td></td>
</tr>
<tr>
<td>DNA (bacterial)</td>
<td>85-88</td>
</tr>
<tr>
<td>Lipoteichoic acids and further Gram-positive bacterial cell-wall components</td>
<td>89-91</td>
</tr>
<tr>
<td>Superantigens</td>
<td>92-94</td>
</tr>
<tr>
<td>Exotoxins</td>
<td>95-99</td>
</tr>
<tr>
<td>Lipoarabinomannans (from mycobacteria)</td>
<td>100, 101</td>
</tr>
<tr>
<td>Fungal components (for example, mannans, glucans, mannoproteins)</td>
<td>102</td>
</tr>
<tr>
<td>Parasite components (for example, phosphoinositol)</td>
<td>103</td>
</tr>
<tr>
<td>Viruses</td>
<td></td>
</tr>
<tr>
<td>Non-microbiological contaminations (for example, cytokines, media, cells, breakdown products)</td>
<td>112</td>
</tr>
<tr>
<td>Solid materials (for example, medical devices, plastic)</td>
<td>113</td>
</tr>
<tr>
<td>Drugs (for example, steroids, bile salts, dapsone, cytokines)</td>
<td>114-116</td>
</tr>
</tbody>
</table>
in humans has not been established, and the mechanisms by which they induce fever are not broadly accepted. For example, a case report of a treatment-associated death has been published (117) that links the fever reaction to the adenoviral vectors used in gene therapy; however, the mechanism of pyrogenicity caused by viruses is still unknown.

In general, the detection of Gram-negative pyrogens (i.e. endotoxins) is not problematic, since they are readily detected by the LAL test. However, substances that inhibit the clotting reaction may mask the presence of pyrogens from Gram-negative organisms. Furthermore, pyrogenic substances from Gram-positive organisms (for example, exotoxins or cell wall constituents) obviously exist, since these bacteria cause severe febrile and septic diseases, often indistinguishable from Gram-negative infections. Rare examples of well-characterised pyrogens of Gram-positive bacterial origin are the superantigens ("pyrogenic exotoxins") of Staphylococcus and Streptococcus species. Consequently, as long as the pyrogenic structures of Gram-positive pyrogens are not sufficiently characterised, they must be considered to be false negatives in LAL tests.

Gram-positive bacteria occur as a special problem in biotechnological manufacturing processes because many of the steps involved are usually contaminated by environmental species in low numbers. Most of them are spore-forming Gram-positive Bacillus species. Recently, tests for the detection of peptidoglycans have been developed (118, 119), but whether they can be used for pyrogen testing has not yet been evaluated or reported.

In addition, mould fungi (for example, Aspergillus niger) represent a frequent contamination of raw materials. Recombinant drugs derived from yeast expression systems also have to be considered. Similar to infections caused by Gram-positive bacteria or viruses, fungal infections often occur as febrile diseases. Fungal pyrogens have not been well characterised so far, except for some mannans, i.e. mannanproteins from the cell walls of Candida albicans.

In conclusion, the chemical nature of non-endotoxin pyrogens is not well characterised. On the other hand, without doubt, drugs must also be free of these contaminants. The clinical significance of non-endotoxin pyrogens is probably underestimated, as their presence is not usually expected. A severe under-reporting has therefore to be anticipated. Since any fever usually occurs several hours after the administration of a contaminated drug, the correlation between this side-effect and the injection is often not recognised. The fact that severely ill patients are usually treated with several intravenous drugs adds to the problem of distinguishing between the course of the disease and any toxic reaction. There is a clear need for a new method for the detection of non-endotoxin pyrogens in vitro. No test means there are no data, but does not mean there are no cases. As shown by the anecdotal observations compiled in Table III, such cases are of relevance in clinical practice.

Problematic test samples

There are a number of products that can create problems in any of the in vivo or in vitro pyrogen tests. The applicability of the tests for the various product classes is summarised in Table IV, and is described in more detail in the following sections.

Problematic test samples for the rabbit pyrogen test

Drugs that influence the central or peripheral mechanisms of body temperature regulation (for example, antipyretic drugs, steroids, dopamine, dobutamine; 74, 120–127), cannot be tested by the rabbit pyrogen test. The same applies to drugs which can cause immunological reactions (for example, immunoglobulins), oily suspensions or detergents. Also, the rabbit pyrogen test cannot be used for cellular preparations, such as blood components and stem cells.

Problematic test samples for the LAL test

Drugs which interfere with the clotting system, i.e. through inhibition (binding of divalent cations such as EDTA, citrate, protease inhibitors) or enhancement (high protein content, proteases), cannot be tested by the rabbit pyrogen test. The same applies to drugs which can cause immunological reactions (for example, immunoglobulins), oily suspensions or detergents. Also, the rabbit pyrogen test cannot be used for cellular preparations, such as blood components and stem cells.

Problematic test samples for the LAL test

Drugs which interfere with the clotting system, i.e. through inhibition (binding of divalent cations such as EDTA, citrate, protease inhibitors) or enhancement (high protein content, proteases), cannot be tested with the LAL test (128, 129). Furthermore, a number of endotoxin-binding components from plasma are known to mask LPS in the LAL test (130). Due to such interference with the test system, many drugs have to be diluted (see below; 131, 132). Turbidity and colour can disturb the measurement principles. Polysaccharides, for example,
from cellulose filter materials, can result in false-positive signals (133). Special problems are evident for solid materials (for example, medical devices, see also below; 134, 135).

Table III: Examples from authors' experience and from the literature of discrepancies between the results of various pyrogen tests and the reactions of patients

<table>
<thead>
<tr>
<th>Product</th>
<th>LAL</th>
<th>Rabbit pyrogen test</th>
<th>Adverse reactions of drug</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulins</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>Author's experience</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>Poole (62)</td>
</tr>
<tr>
<td>Hepatitis B vaccine</td>
<td>-</td>
<td>+</td>
<td>n.d.</td>
<td>Author's experience</td>
</tr>
<tr>
<td>Pertussis vaccine</td>
<td>±</td>
<td>n.a.</td>
<td>yes</td>
<td>Author's experience</td>
</tr>
<tr>
<td>Influenza vaccine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tick-borne-encephalitis vaccine</td>
<td>-</td>
<td>n.a.</td>
<td>yes –1000 cases</td>
<td>Author's experience</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>-</td>
<td>±</td>
<td>yes (&gt; 150 patients)</td>
<td>CDC</td>
</tr>
<tr>
<td>Case report Heidelberg</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>Personal communication</td>
</tr>
<tr>
<td>Various batches of commercial drugs</td>
<td>-</td>
<td>+</td>
<td>yes</td>
<td>Author's experience</td>
</tr>
<tr>
<td>Echinacin</td>
<td>+</td>
<td>-</td>
<td>no</td>
<td>Author's experience</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>Dinarello (61)</td>
</tr>
</tbody>
</table>

- = negative result (pass), + = positive result (fail), ± = inconclusive, n.d. = no data available, n.a. not applicable.
recombinant proteins with cytokine activity, such as IFN-γ or the detection system (for example, rheumatic factors [136]), cannot be tested with in vitro pyrogen tests.

Special problems with pharmaceutical products in the LAL test. There are a number of pharmaceuticals that cannot be tested in the LAL test, such as chemotherapeutics (for example, taxol), suspensions (for example, fluspirilen, prednisolone acetate, corticoids; 74) or liposomes, but most pharmaceutical drugs can be tested at a dilution not exceeding the maximum valid dilution for that product. This can be calculated from the dose of product and the endotoxin limit concentration for the product, as described in the Pharmacopoeias.

With regard to parenteralia, the current Ph.

| Table IV: Comparison of testability of several test samples in different assays |
|------------------|--------|--------|--------|
| **Product** | **Rabbit** | **LAL** | **In vitro pyrogen tests** |
| Drugs with intrinsic problems | | | |
| — metabolic effects | – | + | + |
| — anaesthetics | – | + | + |
| — biological response modifiers | – | ± | ± |
| — recombinant proteins | – | + | + |
| Physical/chemical | | | |
| — pH | ± | + | + |
| — high ionic strength | – | – | + |
| — solubility | – | – | + |
| Cytotoxic drugs | ± | ± | ± |
| Cytotoxic pro-drugs | ± | + | + |
| Cellular products | – | – | + |
| Blood components | – | – | + |
| Blood-derived products | + | ± | + |
| Vaccines | +<sup>a</sup> | ± | + |
| Gene therapeutics | + | ± | + |
| Recombinant proteins | + | + | +? |
| Active pharmaceutical ingredients (API) | – | ± | + |
| Adjuvants | | | |
| — aluminium hydroxide | + | – | + |
| Medical devices | | | |
| — macro | ± | ± | ± |
| — micro | ± | ± | ± |
| Solid materials (filters) | – | ± | ± |

<sup>a</sup> = problematic, ? = not clear, more results necessary, + = testable, ± = limited applicability, relevance not clear.
Eur. monograph (137) stipulates pyrogenicity testing only for volumes higher than 15 ml (dose/day/70 kg) or for parenteralia, which are labelled as "pyrogen-free". Until now, no problems have occurred, but new tests are offering the opportunity to control this situation.

In addition, herbal medicinal products (for example, taxol, ginseng), which can contain up to 40,000 substances in a single preparation, often contain pyrogenic structures (mostly lectins and polysaccharides), as well as components which give false-positive results in the LAL tests (138, 139).

Special problems with biological products in the rabbit pyrogen test and the LAL test:

Vaccines raise a variety of problems with regard to pyrogen testing. Some of them, such as vaccines derived from Gram-negative bacteria, contain endotoxin as a component (140, 141). Some viral vaccines contain inherently pyrogenic components, which are negative in the LAL test (142). Vaccines that contain aluminium hydroxide \( \text{Al(OH)}_3 \) need to be tested in rabbits, since this substance interferes with the LAL test. The interactions of substances in multicomponent vaccines have received little attention. Since vaccines are not for intravenous use, they do not need to be pyrogen-free. Nevertheless, an endotoxin limit is set for many vaccines. Experience with vaccines for veterinary use indicates that the role of contaminating endotoxins in triggering side-effects is underestimated.

There is a strong interference between many blood products and the LAL test, whereas the rabbit test tolerates most plasma derivatives (for example, albumin, factor VIII). However, there are certain products which cause problems in rabbits (for example, immunoglobulins). Some blood products can be tested in the LAL test at a certain dilution. However, the individual compositions of most blood products vary from batch to batch, and from manufacturer to manufacturer.

Problems are quite possible with products from Gram-positive bacteria, such as streptokinase.

Putative new areas for pyrogenicity testing:

- Cellular blood components (platelets stored for up to 5 days at room temperature) are not tested for pyrogens at all (143). This also applies to cellular therapeutics, such as stem cells and lymphokine-activated killer cells.

- Until now, pyrogens bound to a solid phase (for example, medical devices) are probably not completely detected, because only saline rinses of medical devices are tested (predominantly in the LAL test; 144). Endotoxin, and quite possibly other pyrogens, have a high affinity to those products (for example, catheters, coronary stents, hip replacements and contraceptive spirals), and are probably not eluted by saline. Although there are no reports of fever reactions correlated to the use of medical devices in clinical practice, contamination with pyrogens might affect the biocompatibility of these materials (for example, by inducing local inflammatory reactions; 113, 145–148).

- Air-borne pyrogens in stables, the recycling industry, the steel industry, and air-conditioned rooms can affect the respiratory tract (149, 150).

In haemodialysis, there is prolonged contact between large volumes of dialysis fluid and the patient's blood. Pyrogenic contaminants in dialysis fluids or devices may cause fever reactions (151–161).

For several years, high-dosage drug treatments have been introduced for some chronic diseases, such as haemophilia A. On the one hand, the dosages given nowadays are higher than in the past, and on the other hand, a number of patients develop Factor VIII-neutralising antibodies, which in turn necessitate an elevation in the dosage. Therefore, the current safety level for pyrogenicity according to the Ph. Eur. monograph no longer covers this clinical situation. There is a similar situation in high-dosage therapy with immunoglobulins for rheumatic diseases.

Active pharmaceutical ingredients, such as raw materials and bulk materials, which cannot always be tested in the LAL test, often do not allow widespread testing in rabbits, for ethical reasons (162).

In basic research, cytokine-inducing contaminations of cell culture media, FCS or cell culture plastic may cause effects in cell models or diagnostic systems, which are often overlooked (163).

More-complex treatment strategies are continually being developed, which necessi-
tate more-invasive treatments (for example, cell therapy, gene therapy, bone marrow transplantation). These materials cannot be tested in conventional pyrogen/endotoxin tests. Also, the production of biologicals by using recombinant DNA in genetically modified organisms such as Gram-negative bacteria, fungi, mammalian cells and insect cells, is increasing (164, 165). These materials can be contaminated with components from the expression system. While these products can be largely controlled for endotoxins and for viruses, little is known about how to control purification processes to exclude, for example, components of Gram-positive bacteria or fungi. In addition, species-specific products with inherent pyrogenic activity often cannot be tested, except in humans (for example, recombinant cytokines like IL-2, GM-CSF, TNF, immune modulating antibodies such as OKT3 and immunoglobulins [heteragglutins, immune complexes]).

**Novel Pyrogen Tests: Recommendations for Development and Evaluation**

**Parameters**

It is recommended that the terminology and definition of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (166) should be used to describe parameters for the comparison of the test systems. The following should be addressed.

**Theoretical aspects and parameters**

- **Detection limit:** the lowest amount (usually of international reference endotoxin) that can be detected, but not necessarily quantified as an exact value.

- **Qualification:** how the biological indicator system is linked mechanistically to the fever reaction in man or rabbit, respectively.

- **Accuracy:** the closeness of agreement between the value that is accepted either as a conventional true value or as an accepted reference value and the value found. With regard to practicability, this can be done by determination of spike recovery with an endotoxin reference preparation.

- **Precision:** the closeness of agreement (degree of scatter) between a series of measurements in terms of reproducibility (inter-assay) and repeatability (intra-assay).

**Availability of reference materials and controls**

- **Interference inhibition or enhancement by substances other than the pyrogens within the sample.**

- **Range:** the lowest and highest detectable concentrations of a spike with an endotoxin reference preparation.

- **Spectrum of pyrogens:** the variety of pyrogens, other than endotoxins, which can be tested.

- **Limit of quantification:** (for quantitative assays only).

- **Parameters in practice**

- **Robustness:** a measure of the capacity of the test to remain unaffected by small, but deliberate, variations in method parameters, which provides an indication of its reliability during normal usage.

**Readiness/availability of reagents**

- **Test duration:**

- **Versatility:** broadness of applications (pharmaceuticals, biologicals, cellular therapies, dialysis fluid, air-borne pyrogens).

- **Scale-up and automation:** whether the test can be automated for routine use.

**Recommendations for the comparison of novel pyrogen tests with conventional tests**

- **Reference tests**

  - Comparison with both the LAL test and the rabbit pyrogen test are required. For validation, a collaborative study with spiked drugs tested in both the rabbit pyrogen test and in the novel in vitro test appears to be necessary. At least, the potency of the non-endotoxin reference materials used for spiking should be tested in vivo. However, it is most likely that ethical considerations will limit comparison of novel tests with the rabbit pyrogen test.

- **Spectrum of pyrogens to be detected**

  - Microbial pyrogens other than endotoxin, which could occur in a drug — for example, Gram-positive lipoteichoic acid or peptidoglycans, substances of spore formers (mostly Gram-positive) and of fungi (yeast, moulds).
— should be included in these tests. It will be necessary to prepare control samples. The capacity to detect these samples will be tested in comparison with the international standard for endotoxin (the World Health Organisation [WHO] standard). Standards for non-endotoxin pyrogens should be developed, and a calibration in terms of “pyrogenicity units”/IU equivalents for control samples would be useful.

Selection of appropriate test samples

Pyrogen-contaminated samples from hospitals or manufacturers are difficult to obtain. Therefore, intralaboratory evaluation and collaborative studies have to be performed with spiked samples (endotoxin and other pyrogens). The selection of samples should be focused on products which are usually tested in the rabbit pyrogen test. Widely-used pharmaceuticals, such as plasma-derived drugs, vaccines, antibiotics, streptokinase, human serum albumin (HSA), gentamyacin and tetanus toxoid adsorbed on Al(OH)₃, should be tested.

The collection of samples from routine production or hospitals should be initiated, in order to incorporate such samples.

Standardisation of methods and reference materials

Blood and cell lines should be standardised in terms of donors, medium, source, passages, storage conditions, etc. The use of standardised kits (for example, ELISAs for cytokines) would be very helpful with regard to validation and regulatory acceptance. Also, inter-individual differences (for example, polymorphisms) of blood donors or day-to-day variability of cell cultures might need to be monitored on a regular basis. Reference materials are required. It will have to be decided whether non-endotoxin control samples (Gram-negative, Gram-positive and/or fungal) are needed.

Requirements for validation

Any novel in vitro pyrogen test must at least detect endotoxins (the most frequent contaminant) and pyrogens from Gram-positive organisms, as well as, ideally, fungal pyrogens. The spike recovery must be sufficient for a range of products (for example, saline, human serum albumin, vaccine, immunoglobulin G). With regard to non-endotoxin pyrogens, comparison with the rabbit pyrogen test is needed, whereas comparison with the LAL test is sufficient for endotoxins. If available, product batches which failed the rabbit pyrogen test or caused fever reactions in humans should be included in the validation process. It is recommended that the prevalidation and validation of novel pyrogen tests should follow the guidelines established by ECVAM as closely as possible.

Promotion to regulatory acceptance

Testing for pyrogenicity is stipulated by a number of national and international regulatory bodies (for example, the United States Food and Drug Administration [FDA], Washington, DC, USA [167–169], and the European Agency for the Evaluation of Medicinal Products [EMEA], London, UK), organisations (for example, Commune Européenne de Normalisation [170], Organisation for Economic Cooperation and Development, Paris, France, and the WHO, Geneva, Switzerland), and pharmacopoeiae (for example, the Ph. Eur., Strasbourg, France [137, 171, 172], the United States Pharmacopoeia [173–175], and the Japanese Pharmacopoeia [176, 177]). The workshop participants recommended that these institutions should be kept informed of the development of new pyrogen tests, and should be involved in their validation whenever possible. How regulatory acceptance could be achieved in Europe is shown in the following two examples.

European Pharmacopoeia

The European Pharmacopoeia Commission has a strong commitment to replace animal tests by in vitro tests whenever possible. Requests for the replacement of the rabbit pyrogen test by the LAL test in the field of antibiotics (178) and biologicals (179) have led to a revision programme for the corresponding monographs (24 in total). Among the other monographs prescribing the pyrogen test in the third edition of the Ph. Eur. (up to Supplement 2000), there are eight monographs on blood products, seven monographs on dosage forms, eight monographs on excipients, and six monographs on vaccines. Each monograph needs to be examined individually to verify whether the pyrogen test could be replaced by the LAL test or by an in vitro pyrogen test. At present, chapter 2.6.14 Bacterial endotoxins of the Ph. Eur. describes the various methods used to carry out the LAL test, together with guidelines for validating the LAL as a replace-
ment of the pyrogen test. It is foreseen that when a validated in vitro pyrogen test is available, such a method will be included in the Ph. Eur., together with appropriate guidelines for validating the in vitro pyrogen test as a replacement of the pyrogen test.

Procedures for acceptance of the scientific validity of alternative methods within the European Commission

ECVAM and the Environment Directorate General (DG Environment) of the European Commission have jointly developed procedures for acceptance of the scientific validity of alternative methods within the European Commission.

1) The criteria for test development, prevalidation, validation and independent assessment are spelled out in various publications (180–185).

2) The ECVAM Scientific Advisory Committee (ESAC) is kept informed of progress in relation to test development and the planning and conduct of prevalidation and validation studies.

3) When a study (or an independent evaluation of available information about a test method) is completed, the report and conclusions of the management team for the study (or of those responsible for an independent evaluation) are presented to the ESAC, and its members are invited to consider and, where appropriate, endorse the outcome of the study or evaluation.

4) When the ESAC endorses the conclusions and recommendations of a study or evaluation, this is communicated jointly by ECVAM and the DG Environment to other Services of the Commission, and to other appropriate bodies, such as the relevant competent authorities in the Member States of the European Union, the OECD Secretariat, and the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).

5) The other Commission Services discuss the ESAC endorsement with their own advisers and Competent Authorities, and take up a position with regard to the use of the test.

6) The ECVAM/DG Environment Statement and any subsequent statements by other Services of the Commission are published in ATLA; for example, the statements on the scientific validity of in vitro tests for phototoxic potential (186), phototoxicity of UV filter chemicals (187), and skin corrosivity (188, 189), and of in vitro methods for the production of monoclonal antibodies (190).

Conclusions and Recommendations

General

1. The limitations of the rabbit pyrogen test and the bacterial endotoxin test necessitate the development and validation of novel in vitro pyrogen tests.

2. A number of promising novel in vitro pyrogen tests are available, which are based on the human fever reaction, and promise to overcome the problems currently associated with pyrogenicity testing.

3. However, the new tests still have to be validated. An international joint validation study has been initiated (outline given in the Appendix) for this purpose, taking into consideration the recommendations of this workshop.

4. It is essential that organisations and institutions such as Ph. Eur., EMEA, FDA, WHO, etc., are continually informed about, and whenever possible involved in, the development and validation of novel pyrogen tests from the very beginning.

Demands for a novel pyrogen test

5. A novel pyrogen test should not use animals.

6. A novel pyrogen test should be based on the human fever reaction.

7. A novel pyrogen test should detect a broad range of pyrogens (at least endotoxins and pyrogens from Gram-positive organisms and, ideally, fungal pyrogens).

8. A novel pyrogen test should be widely applicable (minimal interference with the products to be tested and suitable also for cellular products and solid materials).

9. A novel pyrogen test should be a quantitative or semi-quantitative method.
10. A novel pyrogen test should be sensitive enough to allow the retrieval of endotoxin spikes at the endotoxin limit concentration listed in the pharmacopoeias for various drugs.

11. A novel pyrogen test should be simple, robust and practicable (the necessary reagents should be available in less-well-equipped laboratories).

Validation

12. It is recommended that the terminology and definition of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use be applied.
13. It is recommended that the prevalidation and validation of novel pyrogen tests should follow the guidelines established by ECVAM as closely as possible.
14. Comparison with the pyrogen tests that are currently used is recommended: comparison with the LAL test is sufficient for endotoxins, whereas the rabbit pyrogen test is at least required for the testing of the non-endotoxin reference materials. The use of the in vivo test should be restricted to a minimum.
15. Microbial pyrogens other than endotoxin, which could occur in a drug — for example, Gram-positive lipoteichoic acid or peptidoglycans, substances of spore formers (mostly Gram-positive) and of fungi (yeast, moulds) — should be included in the validation study.
16. It will be necessary to prepare reference pyrogens. The capacity for detecting these pyrogens should be tested in comparison with the international standard for endotoxin (WHO standard). Standards for non-endotoxin pyrogens need to be developed, and a calibration in terms of "pyrogenicity units"/IU equivalents for control samples would be useful.
17. The intralaboratory evaluation and collaborative studies should be performed with spiked samples (endotoxin and other pyrogens), since pyrogen-contaminated samples from hospitals or manufacturers are difficult to obtain. The selection of samples should be focused on products that are usually tested in the rabbit pyrogen test. Widely used pharmaceuticals should be included, such as plasma-derived drugs, vaccines, antibiotics, streptokinase, human serum albumin (HSA), gentamycin, and tetanus toxoid adsorbed on Al(OH)₃. The collection of pyrogenic samples from routine production or hospitals should be initiated, in order to incorporate such samples. It will have to be decided whether non-endotoxin control samples (Gram-negative, Gram-positive and/or fungal) are needed for routine testing.
18. The standardisation of blood and cell lines in terms of donors, medium, source, passages, storage conditions, etc. is recommended. The use of standardised kits (for example, ELISA for cytokines) would be very helpful with regard to validation and regulatory acceptance. Also, inter-individual differences (for example, polymorphisms) of blood donors, or day-to-day variability of cell cultures, might need to be monitored on a regular basis.

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Pyrogenic reactions in patients receiving conventional, high-efficiency, or high-flux hemodialysis treatments with bicarbonate dialysate containing high concentrations of bacteria and endotoxin. Journal of the American Society of Nephrology 2, 1436-1444.


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validity of the 3T3 NRU PT test (an in vitro test for phototoxic potential). ATLA 26, 7-8.
Appendix 1

Summary of EU project: Human(e) Pyrogen Test (QLRT-1999-00811)

In February 2000, an international joint validation study on advanced pyrogen tests was started. The overall aim of this project, sponsored by the Fifth Framework Programme of the European Commission, is to develop, evaluate and validate a method based on the human fever reaction to replace the rabbit pyrogen test and the Limulus amoebocyte lysate (LAL) test. The network brings together the most promising test systems developed within recent years in Europe, for transnational comparison and subsequent validation of the most promising models. This pre-competitive development should initiate further applications and exploitations for new fields of pyrogen testing, such as cellular therapies, medical devices and pyrogen pollution control in the workplace. A method for introduction into the European Pharmacopoeia (Ph. Eur.) will be developed as a replacement for the rabbit pyrogen test for end-product control. This effort was accomplished by the Ph. Eur., by establishing a group of experts in order to prepare a monograph.

Objectives of the Four Work Packages (WP1–WP4) of the Study

WP1: Evaluation of tests
1) Drafting of Standard Operation Procedures (SOPs).
2) Evaluation of test performance and intralaboratory variances.

WP2: Comparison of Tests I
1) Establishment of each test in two to three partner laboratories.
2) Determination of interlaboratory variation of individual tests; comparison of tests.
3) Selection of the most promising model based on previous results.

WP3: Pre-validation
1) Establishment of the consensus test in all partner laboratories.
2) Prevalidation, development of a prediction model, evaluation of applications.
3) Refinement of the SOP with regard to the analysis procedure; deduction of a prediction model for the rabbit pyrogen test (Partner 8).

WP4: Validation
1) Validation of the test and the prediction model.
2) Definition of applications and limitations of the test; dissemination of results, proposal for a monograph in the Ph. Eur., adoption to industrial need for non-regulated applications.

Project Workplan

The project consists of four work packages, which relate to the four phases of the project (Table I).

WP1/Phase 1: Evaluation of tests

In Europe, in recent years, a number of alternative cellular assays have been developed, which aim to exploit the human primary fever reaction in order to replace the animal test (rabbit pyrogen test) and offer testing in the relevant species (man). Partners 1 to 7 have developed such tests. All of these test systems are based upon the response of human leucocytes (principally monocytes), which release inflammatory mediators (endogenous pyrogens) in response to pyrogenic contamination (exogenous pyrogens). However, the cell-based in vitro assay systems differ with regard to the cells employed (isolated primary blood leucocytes or whole-blood or immortal monocytic cell lines), the mediator determined (interleukin-1, interleukin-6, tumour necrosis factor, neopterin, or nitric oxide), and the precise set-up of the test. Standard protocols for each test are developed or refined and checked by ECVAM for consistency. A set of six test samples is sent to each of the laboratories involved, to compare test performance and intralaboratory variation. Test failures would be investigated at this stage.
**WP2/Phase 2: Comparison and selection of tests**

Protocols are refined again including exchange of endpoints. Each test is transferred to two or three partner laboratories, with documentation of the transfer and checking of transferability. A second set of six test samples is sent to each of the laboratories involved to compare test performance and interlaboratory variation. The most promising test(s) will be selected.

**WP3/Phase 3: Prevalidation**

The selected test(s) is (are) first transferred to an independent laboratory (P9) which was not involved in the development, to test assay transferability and performance without bias. Then the test is established in each of the partner laboratories. A set of 20 test samples is sent to each of the partner laboratories, to permit comparison of this (these) test(s). Minor refinements of the protocol might be considered (1).

**WP4/Phase 4: Validation**

Under blind conditions, a set of ten test samples is sent to each of the partners, and an independent analysis of raw data is performed by Partner 8 according to the method described by Balls & Karcher (2). Based on these results, a final protocol will be published and a method for the Ph. Eur. will be proposed.

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**Table I: Summary of work packages**

<table>
<thead>
<tr>
<th>Work package</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
<th>Phase 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration</strong></td>
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<td>12 months</td>
<td>8 months</td>
<td>10 months</td>
</tr>
<tr>
<td><strong>Number of tests</strong></td>
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<td>4–6</td>
<td>1–2</td>
<td>1–2</td>
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<tr>
<td><strong>Tests per group</strong></td>
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<td>2–3</td>
<td>1–2</td>
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</tr>
<tr>
<td><strong>Number of samples</strong></td>
<td>6</td>
<td>10</td>
<td>20</td>
<td>10 (blind)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td>Protocol</td>
<td>Exchange of endpoints</td>
<td>Analysis procedure</td>
<td></td>
</tr>
<tr>
<td><strong>Deliverable</strong></td>
<td>SOP Intralaboratory variation (reduction of tests)</td>
<td>Transferability Interlaboratory variation (optimisation of tests)</td>
<td>Reproducibility SOP analysis procedure</td>
<td>Validation GLP concordance Draft monograph</td>
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<tr>
<td><strong>Milestone</strong></td>
<td>Optimised protocols; evaluation</td>
<td>Comparison and selection of tests</td>
<td>Prevalidation Validation</td>
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</tr>
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</table>

**SOP = Standard Operating Procedure, GLP = Good Laboratory Practice.**
Network partners and tasks: project leaders and members of the Project Management Committee

Partner 1: Dr T. Hartung, Coordinator Steinbeis Transfer Centre For In Vitro Pharmacology and Toxicology at the University of Konstanz, Germany
Transfer of methodology to industry, Gram-positive and fungal reference materials.

Partner 2: Dr S. Poole NIBSC, London, UK
Test based on isolated primary human blood cells or MonoMac cell lines and IL-6 release, Gram-negative reference materials.

Partner 3: Dr T. Montag-Lessing Paul-Ehrlich-Institut, Langen, Germany
Test based on human whole blood and IL-1 release.

Partner 4: Dr A.M. Gommer RIVM, Bilthoven, The Netherlands
Test based on a human cell line (MonoMac-6) and IL-6 release.

Partner 5: Dr G. Werner-Felmayer University of Innsbruck, Austria
Test based on a human leucocyte cell line (THP-1) and neopterin or NO release.

Partner 6: Dr T. Jungi University of Bern, Switzerland
Test based on a human cell line (THP-1) and TNF release.

Partner 7: Dr P. Brügger Novartis, Basle, Switzerland
Test based on human isolated blood leucocytes and IL-1 release.

Partner 8: Dr S. Coecke ECVAM, IHC, JRC, EC, Italy
Independent biometrical analysis, check of standard protocols, GLP compliance.

Partner 9: Dr I. Aaberge NIPH, Oslo, Norway
Independent evaluation of test performance in phase 3 and 4.

Associate Contractor 1: Dr S. Berthold DPC Biermann, Bad Nauheim, Germany
Provision of ELISAs, kit development.

References