Blood is a quite peculiar juice.
Johann W. Goethe, Faust

Blood alone moves the wheels of history.
Martin Luther

Food for Thought...

The Human Whole Blood Pyrogen Test – Lessons Learned in Twenty Years

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Summary

The whole blood pyrogen test was first described in this journal exactly twenty years ago. It employs the cytokine response of blood monocytes for the detection of microbiological contaminants with the potential to finally replace the still broadly used rabbit pyrogen test. The article reviews its development process, the current status of the test as well as the challenges and missed opportunities. The article highlights the enormous efforts of many people to get the test to where it is today. But it also shows the incredible missed opportunities for implementation and thus sparing about 400,000 rabbits still used for this purpose per year worldwide; in the EU, since the official acceptance of the test, the number of animals used for pyrogen testing did not fall but increased by about 10,000 to 170,000. The test is the first solution enabling adequate pyrogen testing of cell therapies, including blood transfusions, and medical devices, but has not been implemented for either application by authorities. As the test can quantitatively assess human-relevant airborne pyrogens, the contribution of pyrogens to chronic obstructive lung diseases and childhood asthma can for the first time be defined and home and workplace safety improved in the future.

Keywords: pyrogen, endotoxin, alternative test method, monocyte, cytokine

1 Introduction

The term "pyrogen" was coined by Burdon-Sanderson in 1875 for a hypothetical substance in bacteria-free extracts of putrid meat, which caused fever upon injection into animals (for review see Clough, 1951). When Wechselman in 1911 showed that the febrile reactions in patients after injection of Salvarsan (arsphenamine, the substance for which Paul Ehrlich received the Nobel Prize) were due to contamination of the distilled water, presumably by bacteria, this sparked much interest to address this problem. Salvarsan was the first effective treatment for syphilis, the first modern chemotherapeutic agent, but has to be injected as it is not orally bioavailable.

This drug spurred the wide-spread use of injectable drugs and the need for pyrogen control. Hort and Penfeld developed the first rabbit pyrogen test in 1912 and demonstrated that in rabbits Gram-negative bacteria were pyrogenic but Gram-positive very much less so (reviewed in Bennett and Beeson, 1950). We observed the same when we developed a rabbit blood pyrogen test to compare the responsiveness of rabbit and human blood to pyrogens (Schindler et al., 2003), i.e., rabbit blood, like human blood, showed a high responsiveness to LPS but a lower responsiveness to Gram-positive pyrogens than human blood. This difference in rabbit responsiveness between Gram-negative and Gram-positive bacteria, which does not translate to humans, has misled pyrogen research for the last century to

http://dx.doi.org/10.14573/altex.1503241

focus almost entirely on pyrogens from Gram-negative bacteria, i.e., endotoxin also known as lipopolysaccharide (LPS). We might say that this shortcoming of an animal test has tremendously biased our research, with more than 60,000 scientific articles in PubMed on LPS, but only a few thousand on its Gram-positive counterparts.

The whole blood pyrogen test method was first described in ALTEX exactly twenty years ago (Hartung and Wendel, 1995). Shortly before this, a patent was submitted, which formed the basis for the commercial development of test kits. This patent will now expire and it is timely to review two decades of test development. Just to be clear, the author has every possible conflict of interest – this was my baby and it has shaped my personal career to a large extent. Albrecht Wendel, the co-inventor, and I used the respective license fees until he retired at the end of 2008 and I left the European Commission in early 2009 only for financing our laboratories in Konstanz. Since then the author receives license fees from sales of the kit version of the test by Merck-Millipore – the reader is thus advised to take these thoughts with a "grain of salt."

2 The invention – serendipity at work

In late 1994, I was aware of the rabbit pyrogen test (RPT) and its most successful replacement, the Limulus amebocyte lysate assay (LAL) also called bacterial endotoxin test (BET), but not of any need for further alternative methods. Actually, the LAL is one of the economically most successful alternative methods ever, probably topped only by pregnancy tests, which made frog use in pharmacies obsolete. More than 90% of pyrogen testing is nowadays done by LAL, forming an enormous market as recently estimated by MarketsandMarkets[1]: "*The global Pyrogen Testing Market was valued at $462.38 million in 2014 and poised to grow at a CAGR* [compound annual growth rate] *of 12.23% between 2014 and 2019, to reach $823.14 million in 2019*." Given the roughly ten times lower cost of LAL compared to rabbit testing, this means about 80% of this is the LAL market.

In November 1994, I attended a small conference "*Replacement, Reduction and Refinement of Animal Experiments in the Development and Control of Biological Products*" at the Paul-Ehrlich-Institute, the German control authority for blood products and vaccines, where I was invited to talk about my liver cell co-culture model, an alternative method to study inflammatory processes and the result of my PhD in 1991 (Hartung and Wendel, 1991). On this occasion, I learned that the LAL was not applicable to many vaccines as the very common adjuvant aluminum hydroxide, an additive boosting the immune response, interferes with LAL testing, and that many pyrogenic components of vaccines are not covered by LAL, which detects lipopolysaccharides from Gram-negative bacteria only. I much later learned that the LAL also reacts with glucans, which are common in fungi but also, e.g., cellulose. These structures, which are not pyrogenic for humans, pose problems for drug testing by LAL, resulting in false-positive signals when cellulose filters are used in the drug production process. It was very satisfying to later see that the whole blood assay indeed allows testing of these vaccines (Fischer et al., 1998; Carlin and Viitanen, 2003, 2005; Stoddard et al., 2010), which originally prompted my idea.

I saw a solution at hand: to employ a test I had been using over the last few years, a human whole blood cytokine release assay to detect human-relevant pyrogens. I came across this type of assay in a publication by Desch et al. (1989). Later I found out that Kirchner et al. already described a similar test in 1982, to the best of my knowledge the first whole blood cytokine release assay. I had been using this approach for my research *in vitro* and, I think, as the first investigator from 1992 on also as an *ex vivo* assay in blood of human volunteers and patients undergoing immune modulatory treatments or with diseases. A key publication arising from the *ex vivo* application was a study sponsored by Amgen (Hartung et al., 1995), in which we used the assay to study the immune effects of Neupogen (recombinant granulocyte colony-stimulating factor, G-CSF) in 21 healthy volunteers. This required a broader characterization of the test, which showed some remarkable features: It was very easy to perform, handling only liquids and without the need to perform typical cell culture procedures; there was no detectable cytokine release in the absence of stimulation; the response to endotoxin was concentration-dependent and very reproducible on different days with the same untreated donor and between different individuals; cytokine release could be induced with various immune activators, which we could call pyrogens or nowadays often pathogen-associated molecular patterns (PAMPs). When hearing about the problems of pyrogen testing for vaccines, it instantly occurred to me that the whole blood cytokine release assay would lend itself as a solution. I discussed this in the evening with my friend Franz Gruber, at the time editor of ALTEX, over a glass of wine or more… He instantly said "*Write this up for ALTEX!*" I did, and this is how things started (Hartung and Wendel, 1995; English translation Hartung and Wendel, 1996).

> ***First lesson: Researchers need to know about the needs of applied sciences in order to bring their science to implementation. Academics often do not realize what goodies they have on their shelf.***

The next step was to convince our department head, my long-term mentor and friend, Albrecht Wendel, to patent the new application. As pharmacologists we learned early enough that no idea will ever make it to market if it is published and not patented. In retrospect it is clear that we would never have gained the support of the different companies that licensed the test and the whole development of the test likely would have been abandoned at some point without the patent.

[1] http://www.marketsandmarkets.com

Second lesson: Patenting new methods is critical for their marketing, which gets them standardized, internationally available and visible. Premature publishing cancels this important driving force. The endurance required to attain optimization, validation and acceptance needs such drivers.

The actual innovation of the whole blood pyrogen test might be considered minor: Whole blood cytokine release assays had been described more than a decade earlier (see above); as I learned myself only then, the idea to detect pyrogens using monocyte activation (using isolated PBMC, peripheral blood mononuclear cells) was proposed first 10 years earlier by Charles Dinarello (Dinarello et al., 1984) using the rabbit (!) as a read-out and was later refined by Stephen Poole and co-workers (Poole et al., 1988a,b) who used an ELISA to detect the cytokines. At the time, these publications led to discussions in the different pharmacopoeias on a humanized pyrogen test. But these were not the first, as I much later found a publication by Duff and Atkins (1982), who described a similar approach and already then compared their results with the LAL.

Thus, the innovation was, in retrospect, just the combination of two well-established components, the use of monocyte activation for pyrogen detection and the whole blood cytokine release assay. But it was enough to convince the patent authorities in Europe, the US and Japan. Thomas A. Edison is quoted for "*Genius is one per cent inspiration, ninety-nine per-cent perspiration*." This does not mean to propose that the assay was a matter of genius, but to illustrate that two decades

Tab. 1: Patents associated with the whole blood pyrogen test

Patent applicant(s)	Patent title	Patent numbers
Hartung and Wendel	Test for determining pyrogenic effect of a material	EP 0 851 231 B1 granted 15.12.1997 (countries: AT, BE, CH/LI, DE, ES, FR, GB, IT, NL, SE), US CIP 10/761,237 submitted 23.12.1997, granted 06.04.1999, JP 9-354572 submitted 24.12.1997, granted 04.04.2008
Hartung and Wendel	Test procedure with biological system	EP 0 741 294 B1 submitted 24.04.1996, granted 05.11.2003 (countries: AT, BE, CH/LI, DE, ES, FR, GB, IT, NL, SE), USP 5 891 728 submitted 02.05.1996, under evaluation, JPP 3 667 439 submitted 07.05.1996, granted 15.04.2005
Patel and Poole	Pyrogenicity test for use with automated immunoassay systems	US 09/727,561, EP1234181A1, EP1234181A4, EP2295968A2, EP2295968A3, EP2295968B1, US20010034037, WO2001048481A1, WO2001048481A9, submitted 01.12.2001
Hartung	Method for assaying flowing media for microbial toxins	EP 02 729 818.9 submitted 25.03.2002, under evaluation, US 10/474 694 submitted 10.10.2003, granted 29.06.2010, JP 2002-581 998 submitted 04.12.2003, granted 10.04.2009 (discontinued 2013)
Hacket, Hartung, Otto, Schön, Zimmermann	Method for determining the content of endotoxins in liquids	DE 10247430 A1 submitted 11.10.2002, PCT/EP2003/011028 submitted 06.10.2003
Mueller, Montag-Lessing, Spreitzer, Loeschner, Schwanig	Method for the cryopreservation of human blood	WO2007054160 A2, EP1787512A1, submitted 28.09.2006
Montag-Lessing, Spreitzer, Loeschner	Cytokine-based pyrogen test	WO2007107370 A1, EP1837658A1 submitted 22.03.2007
Löschner, Montag-Lessing, Spreitzer	Method for the production of frozen blood or frozen blood cells for biological assays	WO2008037481 A3, CA2683874A1, CN101583268A, EP1908346A1, US20090317790, WO2008037481A2, WO2008037481A3 submitted 27.09.2007
Montag-Lessing, Spreitzer, Loeschner	Improved method for the production of frozen blood or frozen blood cells for biological assays	CA 2683874, CN101583268A, EP1908346A1, EP2068620A2, US20090317790, WO2008037481A2, WO2008037481A3 submitted 27.09.2007
Poole, Patel	Monocyte activation test better able to detect non-endotoxin pyrogenic contaminants in medical products	US 20100203551 A1, CA2633523A1, CN101346630A, CN101346630B, CN103901208A, EP1977252A1, EP1977252B1, EP2360479A2, EP2360479A3, EP2360479B1, US7736863, US8053200, US8058021, US2007018 4496, US20100203557, US20120058559, WO2007076411A1, submitted 26.04.2010
Hartung	Method to prepare a reference material for pyrogen testing in cell and particle preparations	Provisional application of 08.07.2014

of work by many people were necessary to turn that first idea into a success.

A number of patents by others and us followed (Tab. 1). The discussion whether patented methods can be used for regulatory purposes, as they would create a monopoly, has gone on a long time. Initially, OECD would not produce test guidelines with patented methods but then found a way around this dilemma by defining performance standards that follow-up products need to meet in order to satisfy the guideline. We had similar discussions in ECVAM about the validation of patented methods (Linge and Hartung, 2007). In the end, we were not concerned in the case of the whole blood assay, because we always promoted in parallel the other non-patented variants of the test. Thus, an end-user would always have the choice to use non-patented variants, and patents do not limit the research use of tests anyway.

3 The importance of commercialization

The idea of the whole blood pyrogen test was intriguing enough to find, in continuation, a number of licensors, who produced kits (Tab. 2), as well as an in-house license by Hoffmann-LaRoche. The test kits were virtually identical as they each contained the exact same ELISA, endotoxin reference materials that were calibrated against the international standard, and Gram-positive lipoteichoic acid (LTA) from my own laboratory. Biotest / Merck-Millipore for the first time also made cryopreserved blood available as an add-on to the test. The cryopreservation method was patented by Albrecht Wendel and myself one year after the original patent in 1996 (Schindler et al., 2004, Tab. 1).

Various monocyte activation tests (MAT), summarized in Hartung et al. (2001), which are based on the understanding of the human fever reaction and ELISA technologies (Poole et al., 1988a,b; Hansen and Christensen, 1990; Werner-Felmayer et al., 1995; Eperon et al., 1997; Moesby et al., 1997; Gaines Das et al., 2004) were developed in addition to the whole blood pyrogen test variants. All MAT detect Gram-negative endotoxin (lipopolysaccharide, LPS), non-endotoxin pyrogens (NEP) and mixtures, but are based on different test systems, using either human whole blood, cryopreserved blood, peripheral blood mononuclear cells (PBMCs), monocytic cell lines (MONO MAC 6, MM6) or the human acute monocytic leukemia cell line (THP-1) as a source for human monocytes, and various read-outs were established. Table 3 shows a comparison of the different MAT approaches. Noteworthy, none of the other MAT tests has been commercialized yet, though new cell line-based assays based on the transfection of pyrogen receptors have been patented[2] and such cells are commercially available[3].

Disappointingly (from an economical, a product safety and an animal welfare aspect), the MAT have taken over – though accepted by regulators in Europe and the US for more than five years – far less than 1% of the pyrogen test market. The reasons are that no other MAT has been marketed, that the marketing of the whole blood MAT was limited and that the use of accepted alternatives was never enforced by regulators. It is quite incredible that, after the acceptance of the MAT, rabbit use for pyrogen testing even increased by about 10,000 (comparing 2008 to 2011 figures) to more than 170,000 rabbits used in Europe per year. This increase is owed to the fact that the European Pharmacopoeia introduced a pyrogen test requirement also for injectables below 25 ml – many of these are lipophilic and cannot be tested in LAL, and thus are tested in the RPT. Noteworthy, the whole blood test can handle these test materials (Schindler et al., 2006b), but apparently is not being applied to an appreciable extent. This number of 170,000 rabbits per year must be compared to just 5,000 rabbits per year for the Draize rabbit eye and the Draize rabbit skin irritation test together. This means that since its regulatory acceptance (and

Tab. 2: The kit versions of the whole blood pyrogen test

Company	Kit Name	Time
DPC Biermann, Bad Nauheim, Germany	Pyrocheck	1996 - 2000
Charles-River Endosafe, Charleston, US	Endosafe IPT (In-vitro Pyrogen Test)	2001 - 2008
Biotest, Dreieich, Germany	PyroDetect	2009 - 2011
Merck-Millipore, Merck KG, Darmstadt, Germany	PyroDetect	2012 -

Tab. 3: Advantages and disadvantages of different MAT variants

Source of monocytes	Advantage	Disadvantage
Cell lines	– No donor	– High variability – Laborious
PBMC	– Most sensitive	– Handling artifacts – Infection risk
Fresh whole blood	– Cell suspension – Physiological – Strongly buffered	– Infection risk
Cryopreserved whole blood	– Physiological – Standardization – Commercial kit available (PyroDetect system, Merck Millipore)	– (DMSO present)

[2] http://www.google.com/patents/US20090253134

[3] http://www.invivogen.com/docs/Insight200609.pdf

there is no product that has yet been shown not to be testable with the whole blood MAT), more than 800,000 rabbits have been used without any sensible need, but nobody cares. Validation bodies, the European Commission and other adamant promoters of alternative methods close the books and feel they are no longer responsible once a method has been validated and accepted. This is a grave oversight continuing despite the new EU Directive 2010/63/EU on the use of animals for scientific purposes (Hartung, 2010).

Third lesson: Even when they have overcome the hurdles of development, validation and acceptance, implementation of alternative methods is slow due to the lack of enforcement. Nobody urges companies to apply accepted alternative methods.

4 The long road to acceptance – 15 years from development to acceptance is too long

As an academic researcher in the mid-nineties, I was pretty naïve about the acceptance process for an alternative method. Some validation seemed necessary, but otherwise promising animal welfare legislation in Europe requesting the use of an alternative method if reasonably available seemed to be an easy way to reach implementation.

The immediate interest by the European Pharmacopoeia was very promising: The chair of the pyrogen working group, Dieter Krüger, initiated an independent evaluation and financed this via Roche / Boehringer-Mannheim. Markus Weigandt (with help from Peter Lexa and Michael Jahnke) carried out a thesis at the University of Heidelberg supervised by Hans-Günter Sonntag. Some highlights of his results[4]: Many false-negative results for LPS-spiked samples in the rabbit assay were detected correctly in the human whole blood assay; of 84 blood samples from healthy donors none displayed spontaneous cytokine release but all reacted to LPS in a homogenous manner with inter-individual absolute amounts of interleukin-1 (IL-1) release; the test was positive for *Mycobacterium terrae*, a Gram-positive bacterium; the limit of detection (final concentration) was in the low pg range for LPS, i.e., similar to LAL; 27 commercial drugs showed different forms of interference, which in all cases could be overcome by dilution to correctly identify the established LAL endotoxin thresholds – no false-positives or false-negatives; studying an iron chelating drug, for the first time pyrogen detection in a commercial drug not testable in rabbit or LAL tests was demonstrated. Markus Weigandt concluded (my translation)[5], "*These study results prove that the HVP* [human whole blood pyrogen test] *represents a sensitive, reliable pyrogen test for drugs suitable for routine use, which is clearly superior to both pharmacopoeial methods*." Some of these thesis results were published in scientific articles (Weigandt et al., 1998; Jahnke et al., 2000).

Already early on, the test was enthusiastically supported by the late Thomas Montag-Lessing at the Paul-Ehrlich-Institute (PEI). He, together with Matthias Fischer in the beginning and in continuation Ingo Spreitzer, who changed into Thomas Montag-Lessing's group after his PhD with me, was a "partner in crime" for most of these twenty years. His group continued to evaluate, optimize and adapt the test for regulatory uses (Montag et al., 2007; Fischer et al., 1998; Spreitzer et al., 2002, 2008).

The development and validation of the test, for many years coordinated by Stefan Fennrich in our group, was supported by a number of funding bodies with a total of about €5 million. These included the European Commission ("Human(e) Pyrogen Test" in FP5, "Spiderman" in FP6 and "Silkbone" in FP7), the German Ministry for Education and Research (BMBF), the German Ministry of Economy and Technology (BMW), the Federal State of Baden-Wuerttemberg, the German National Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET), the Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing (SET), the German Academic Exchange Service (DAAD), the Swiss Foundation 3R and the European Space Agency (EADS) among others; an at least similar investment by the licensing companies added to this.

The first hurdle was validation. It turned out that the European Centre for the Validation of Alternative Methods (ECVAM), at the time headed by Michael Balls, was interested, and with Sandra Coecke and Marlies Halder I found early partners in this. However, there was no funding available for the necessary ring trials. Thus, I put together a consortium representing all recent European proposals for novel pyrogen tests and applied for funding with the European Commission, with success[6]. The study partners included beside ECVAM and ourselves at the University of Konstanz, four national control authorities (the British NIBSC, the Norwegian NIPH, the Dutch RIVM and the German PEI), Novartis as an industrial partner and two academic partners (University of Berne, Switzerland, and Innsbruck University, Austria); the European Pharmacopoeia joined us as an unfunded associated partner. This validation (Hoffmann et al., 2005a) and a catch-up validation for the cryopreserved MAT variants (Schindler et al., 2006a), which included also the small start-up company Qualis, Konstanz, Germany, was a major success, leading to validity statements, a press conference of the European Commissioner for Research Philippe Busquin and follow-up evaluations by the different validation bodies worldwide. This three-year validation program was a most interesting learning experience: Obviously, the different proponents all came with their own assays but they had to work together, be the second and third laboratory for others, and increasingly the direct comparison helped everyone to appreciate the advantages of

4 http://archiv.ub.uni-heidelberg.de/volltextserver/2177/1/ZusammenfassungWeigandt.pdf

5 "Mit den Untersuchungsergebnissen konnte der Nachweis erbracht werden, dass mit dem HVP ein sensitiver, zuverlässiger und für den Einsatz in der Routine geeigneter Test zum Pyrogennachweis in Arzneimitteln vorliegt, der den beiden Arzneibuchmethoden deutlich überlegen ist."

6 http://cordis.europa.eu/project/rcn/51987_en.html (last accessed March 21, 2015)

Tab. 4: Specific characteristics of whole blood and cryo-blood assays

Whole blood assays	Cryo-blood assays
Blood donor dependent, primary cells, no isolation, cell suspension (Daneshian et al., 2009)	Standardized and pretested primary cell pool, no isolation after thawing, cell suspension (Schindler et al., 2004)
Internationally validated (Hoffmann et al., 2005a)	Internationally validated (Schindler et al., 2006a)
Saline, thermoblock incubation possible	Culture medium, thermoblock incubation possible
Extensive supportive data (90 different LPS, 40 LTA, 50 fungal spores, 5 exotoxins, etc.), incriminated samples and > 80 products, direct comparisons to rabbit testing; not disturbed by adjuvants	Supportive data for various non-LPS pyrogens and routine product testing
Variants: commercial kit available[1], medical devices/solid materials (Hasiwa et al., 2007), airborne pyrogens (Kindinger et al., 2005), adsorb/wash-extraction/enrichment (Daneshian et al., 2006, 2008), cell therapies (Montag et al., 2007), one-plate assay (Poole et al., 2003)	Variants: see whole blood assays, storage at -80°C or liquid nitrogen

[1] http://bit.ly/1xB1WmS

others' methods and to see the problems of all. We had the most problems with the cell line assays, which sometimes worked extremely well, but at other times failed for months in a given laboratory: The two THP-1 cell systems did pretty well in the prevalidation, but one and two laboratories, respectively, failed completely in the final validation and no catch-up work was able to amend this. Just the opposite, the MonoMac-6 test simply did not work and we were about to drop it from the study several times but did not want to loose our Dutch partners and their expertise. In the final validation it suddenly had excellent results in all three laboratories. This experience is one of the reasons for my personal skepticism regarding the regulatory use of tumor cell lines (Hartung, 2007, 2013). They require too many controls for each and every experiment and too often an entire test set must be disregarded. A major factor for success of the validation studies was the direct involvement of ECVAM, the European Pharmacopoeia and four national control authorities, which prevented the project from becoming an academic playground.

The validation study and parallel work in a number of laboratories and collaborations highlighted the characteristics of the whole blood assay with fresh or cryopreserved blood (Tab. 4) (Schindler et al., 2009; Hasiwa et al., 2013a,b). Some independent evaluations were very favorable (Pool et al., 1998, 1999; Nakagawa et al., 2002; Andrade et al., 2003; Francois et al., 2006; Kikkert et al., 2008; Megha et al., 2011; Banerjee and Mohanan, 2011). On the way, the work around the whole blood assay received several awards (Tab. 5).

But it was a sour lesson to see how long the peer-review and acceptance process lasted: The validation study ended in January 2003, but it took until 2006 to achieve validity statements in Europe. A similar recommendation was made by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2008, 2009). The big moment: In 2010, the MAT Monograph 2.6.30 was implemented into the European Pharmacopoeia (EDQM, 2010) and since then can be employed as a substitute for detecting Gram-negative endotoxins and NEPs alike in injectables on a case-by-case basis. The same position, but restricted to endotoxins, was adopted by FDA (2009). The most recent guidance[7] states that firms producing products for which pyrogen testing is required may use alternative methods if they provide advantages in terms of accuracy, sensitivity, precision, selectivity

Tab. 5: Awards received for the whole blood pyrogen test

Year	Awardee(s): Title of Award
1996	Hartung and Wendel: Doerenkamp/Zbinden Award for replacement of animal experiments
2001	Hartung and Wendel: Business Innovation Award of the region Lake Konstanz for the development of an alternative pyrogen test
2002	Hartung: RIVM Award at the World Conference on Animal Use and its Alternatives
2003	Hartung, Fennrich and Wendel: Environment Award of the Landesbausparkasse Baden-Wuerttemberg
2004	Hartung and Wendel: Steinbeis Technology Transfer Award
2006	Daneshian and von Aulock: Environment Award of the Landesbausparkasse Baden-Wuerttemberg
2011	Fennrich, P. Wendel and A. Wendel: 365 Landmarks in the Land of Ideas Award
2013	Rivera-Mariani: LUSH Young Investigator Award

[7] http://www.fda.gov/drugs/guidancecomplianceregulatoryinformation/guidances/ucm314718.htm (last accessed 21 Mar 2015)

or adaptability to automation or computerized data reduction, and in other special circumstances. Alternative methods should be subjected to appropriate validation and shown to achieve equivalent or better results compared to the standard method. The document also provides guidance for transitioning from one test method to another.

The EMEA encourages the replacement of the RPT by alternative tests such as LAL or MAT in plasma-derived medicinal products (EMEA, 2009) demanding: "*The EMEA encourages every effort to further develop and establish this type of alternative test*." The MAT also found acceptance in countries such as Brazil and Cuba, but not, for example, in Japan. The JaCVAM Regulatory Acceptance Board determined[8] on the basis of the ECVAM and ICCVAM assessment:

> *"These test methods are based on an endotoxin-induced pyrogenic mechanism that is scientifically valid in principle. The validation studies present results that exhibit a high correlation with the rabbit pyrogen test, but it remains necessary to increase the number of compounds and implement validation at multiple laboratories before these test methods can be adopted as an alternative test method. At present, these test methods are not suitable as a replacement for endotoxin tests. Given that JP15 makes allowances for the move from test methods that use rabbits for detection of pyrogenic substances to those that test for endotoxins, these test methods afford only limited value in Japan. We recognize potential for these test methods, but do not find them to be immediately valid alternatives at this point in time."*

From my point of view, this statement lacks a stringent differentiation between scientific validity and regulatory use. It is fine that the Japanese Pharmacopoeia relies on LAL, or more precisely mainly on the Japanese variant, the *Tachypleus* amoebocyte lysate assay (TAL), and sees no need for another endotoxin test. This however does not mean that the new assays are not valid. In a globalized pharmaceutical market, this represents a roadblock.

However, we have encountered a preference for LAL / TAL by regulators and regulated industries throughout these two decades, which is at first sight understandable: The LAL is cheaper, faster and almost 30 years older, with all the experience and level of standardization developed in fifty years. Only when specific problems arise that impede transition from the rabbit test or would require returning to the RPT, an interest in MAT arises.

It is therefore necessary to address the shortcomings of the rabbit test and the LAL. Astonishingly, there is no open discussion about these shortcomings, such as review articles, etc. Probably the desire to move away from rabbit testing and the commercial pressure of the LAL industry were too strong to really stress the problems of LAL. Given that Good Manufacturing Practices today almost always result in pyrogen-free products, the desire to avoid animal use is very understandable. I was very much impressed when the person responsible for pyrogen testing at the time at Behring, Germany, reported at a meeting that an analysis of their last 10,000 rabbits used showed not a single positive sample.

This actually makes the product-specific validation to transition from rabbit tests to LAL rather simple: Generally, it is requested to show equivalent results for three batches (simple as they are negative) and the lack of interference of an endotoxin spike. And here lies a major fault: The spike is typically done just before testing. The masking of endotoxins, i.e., their binding to compounds, making them invisible for the LAL, takes some time. The LAL succeeds to retrieve the LPS added, e.g., to human serum albumin, only when the test is carried out shortly after spiking. The phenomenon has recently been addressed by the German Hyglos company[9], a new kid on the block of pyrogen testing. For recombinant proteins, this problem has been nicely addressed by Wakelin et al. (2006), showing that whenever an immune mediator or other protein shows endotoxin-like properties, these probably stem from an endotoxin contamination.

Fourth lesson: A substantial number of products might have transitioned from rabbit testing to LAL because of flawed product-specific validations (not allowing time for endotoxin masking).

Foster Jordan, the CEO of Charles-River Endosafe, the largest LAL manufacturer, once told me that 90% of LAL testing is done on water samples. Which species of bacteria are found in water? *Escherichia coli* is found only in low quality supplies, not typically in drinking water in industrialized countries. In contrast, *Pseudomonas aeruginosa* bacteria are ubiquitously found in the environment. Because of their ability to grow in water at 4°C, they are a major source of contamination in purified water, especially dialysis solutions or water prepared for injection purposes (Capelli et al., 1993; Sundaram et al., 1996). When we compared the potency of different LPS in the whole blood assay and LAL, it was most interesting that Pseudomonas LPS was 1,000fold less potent in inducing cytokine release than LPS from *E. coli* or *Salmonella* (in line with the doses needed to induce fever in rabbits), but equipotent in LAL (Dehus et al., 2006). This means we might dramatically overestimate the pyrogenic burden of many water samples using the LAL.

From two decades of experience and direct comparisons, a list of problems of the LAL and a comparison with the MAT was compiled (Tab. 6). The comparison with the RPT is further extended in the next chapter.

A real eye-opener came when, in parallel to the validation study of the MATs, the LAL was carried out by two of the participating national control authorities: The LAL performed by the Norwegian Institute of Public Health (NIPH) on the same samples used in the MAT tests resulted in 67% sensitivity and 100% specificity and the same test done by the UK National In-

8 http://www.jacvam.jp/files/doc/13_01/13_01_Ben1.pdf (last accessed 21 Mar 2015)

9 http://www.endotoxin-test.com/wp-content/uploads/2013/10/Low-Endotoxin-Recovery-in-Common-Protein-Formulations.pdf; http://www.endotoxin-test.com/wp-content/uploads/2015/02/Workshop_Endotoxins-WS_Introduction_WMutter-hyglos.pdf

stitute for Biological Standards and Controls (NIBSC) showed a sensitivity of 83% and a specificity of 33% (Hasiwa et al., 2013b), not as good as the MAT results.

Fifth lesson: MATs overcome often neglected problems of the LAL – they are not only the solution for those products / applications for which we cannot transition from rabbits to LAL.

5 Alternative methods can outperform animal tests

Alternative methods are typically expected to be almost as good as the reference test, in our case the rabbit or LAL pyrogen test. The response of human cells, horseshoe crab amebocytes (Petri and Fennrich, 2000) and rabbits (Schindler et al., 2003) to Gram-negative endotoxin has been studied extensively, and the comparison reveals a good correlation of the whole blood test with the RPT, with results correlating with the content of pyrogens in the sample (Fennrich et al., 1999a,b; Hartung et al., 2001; Hartung, 2002; Schindler et al., 2009). Both RPT and whole blood MAT showed 3-4 log-order differences in potency of different LPS samples. Notably, no such differences in potency were observed for the LAL, in which different LPS differed by a maximum of one log-order in their potency (Fennrich et al., 1998).

Five variants of the MAT have been standardized and validated (Hoffmann et al., 2005a; Schindler et al., 2006a) by ECVAM and the ECVAM Scientific Advisory Committee (ESAC) as alternatives to the RPT for endotoxin pyrogen detection[10] (ECVAM, 2006), and they proved to have a lower detection limit than the rabbit test (Hoffmann et al., 2005b). They are more accurate, cost-efficient and more time-efficient. They are also able to detect Gram-positive pyrogens and therefore meet

Tab. 6: Limitations of rabbit pyrogen tests and LAL versus the MAT

Rabbit pyrogen test	**MAT**
Animal use	None
Costly and laborious	Less than 10% of rabbit test costs
Species variations, strain variations	None, remarkably low inter-individual differences
Examined only for endotoxins	Broad spectrum of pyrogens studied
Pass/fail test	Quantitative test
High variability resulting in many repeat tests	Low variability
Stress affects body temperature	No
Replicate experiments (animal re-use is quite common)	No
No positive controls	Positive controls are part of protocol
Test is not applicable for certain drugs: Radiopharmaceuticals (short half-life), Chemotherapy (toxic), sedatives/analgesics (lower body temperature), Cytokines (increase body temperature), Antibiotics/plasma proteins/antigens (allergic reactions)	So far all assessed drugs were testable with appropriate dilution; the adsorption-wash-in-vitro-pyrogen-test was developed for cytotoxic substances (Daneshian et al., 2006)
LAL	**MAT**
Restricted to endotoxins	Coverage of Gram-positive (Hasiwa et al., 2013a) and fungal pyrogens (Schindler et al., 2009)
Disturbed by endotoxin-binding components (often not seen because of spike just before testing), e.g., blood components, lipids, solid materials (medical devices), cell therapies	Not observed
Potencies of pyrogens and synergies not reflected; difficult to correlate with rabbit test	Correlates with rabbit (Fennrich et al., 1998)
False-positive for glucans, cellulose and many herbal preparations	No
Not a full *in vitro* substitute; US landings down from 3 to 0.7 million (1998-2005), see also (Anderson et al., 2013)	No animal use or suffering

[10] Statement on the validity of in-vitro pyrogen tests. https://eurl-ecvam.jrc.ec.europa.eu/about-ecvam/archive-publications/publication//ESAC24_statement_pyrogenicity_1.pdf (last accessed March 27, 2015)

the quality criteria for pyrogen detection, as defined in the recommendations of an ECVAM workshop report (Hartung et al., 2001). It could be concluded that the MAT provides reliable and reproducible results for many final products (Jahnke et al., 2000; Spreitzer et al., 2002; Andrade et al., 2003). In fact, I am not aware of a single product, which could not be tested after either diluting or employing other adaptations of the protocol.

When we obtained data on 171 rabbits, which were tested as controls by the Paul-Ehrlich-Institute, we found that the variability of rabbit responses was impressive, resulting in 58% specificity and 88% sensitivity for the rabbit to detect endotoxin in saline (!!!) (Hoffmann et al., 2005b). Any substance interference would further lower these values.

Sixth lesson: Tests based on human-relevant mechanisms and measuring biomarkers of pathophysiology can be better than animal tests. They are also often enabling technologies, which allow uses not possible with the animal test.

The whole blood MAT (Schindler et al., 2009) has the advantage that no cell culture is required and no preparation artifacts occur. The cells are kept and maintained in their natural environment, i.e., plasma. In contrast to the LAL, the whole blood test reflects the potency of different LPS species in the rabbit (Fennrich et al., 1999a). Since a cell suspension is used, blood as a reagent can be brought into contact with any material, including medical devices (Hasiwa et al., 2007; Mazzotti et al., 2007) or filters loaded with specimens from air samples (Kindinger et al., 2005). In contrast, cell lines in the validation study showed drifts and shifts in their responsiveness to pyrogens, letting MonoMac-6 cells fail the prevalidation and the two THP-1 tests fail the validation phase. With the introduction of cryopreserved pooled human whole blood (Schindler et al., 2004), which is pretested according to the standards used for blood transfusion, concerns about availability, donor differences, and infectious threats have been overcome. It has also been adapted to rabbit blood, allowing the assessment of species differences in pyrogen detection (Hartung et al., 1998; Schindler et al., 2003). The test is not disturbed by several components that prohibit LAL or RPT testing, such as aluminum hydroxide in vaccines (Carlin and Viitanen, 2005), lipidic parenterals (Schindler et al., 2006a), toxic or immunomodulatory drugs (Daneshian et al., 2006), water and dialysis solutions (Daneshian et al., 2008), and herbal components with glucan-like structures (Daneshian et al., 2006). It is the only MAT for which standardized kit versions are internationally available. For these reasons it is more broadly used and more data are available than for other MAT variants.

6 The non-endotoxin pyrogen safety gap

There is no doubt that microorganisms other than Gram-negative bacteria produce fever. However, the evidence for inorganic non-microbiological compounds producing fever is actually rather weak as too often no proper exclusion of microbiological contaminations was carried out. However, it is generally believed that inorganic particulate matter and organic dust can induce inflammatory reactions.

We addressed the question of non-endotoxin pyrogens quite comprehensively in a background review paper (Hasiwa et al., 2013a,b). There is clear evidence of contaminated drugs that are negative in LAL and sometimes also in the rabbit test but can be found with the whole blood test. These are only a few case reports, but this does not mean that this is a rare phenomenon:

- Reporting of side-effects to authorities by physicians is notoriously bad.
- Parenteral medicines are given to very sick patients, who often have fever already, are receiving fever-reducing medicines, or the cause of fever is difficult to identify because of too many disease- and treatment-related aspects.
- The fever reaction occurs with a delay of 1-2 hours.
- The incriminated batches of drug are proprietary and the companies typically do not agree to further studies, which would link their product to contaminated batches.
- The stability of non-endotoxin pyrogens is not known, which might lead to discrepant results in later tests.

The true extent of the non-endotoxin pyrogen (NEP) safety gap will only become clear when larger screening by MAT is introduced. It is clear that all MAT variants are capable of detecting more than LPS and that these other pyrogens are relevant. However, instead of seizing the opportunity and monitoring products, this is turned against the novel tests, when validation for NEP is requested for acceptance of MATs. Spreitzer et al. (2008) phrased it nicely: "*It is remarkable that the Limulus-Amebocyte Assay (LAL; Bacterial Endotoxin test; only capable of detecting Endotoxin) was good enough to replace the rabbit pyrogen test for many products whereas the acceptance for alternative pyrogen tests is delayed because they are officially only validated for the detection of Endotoxin until now*."

For my own group the whole blood assay formed the basis to isolate and study non-endotoxin pyrogens in about 120 publications led by Siegfried Morath, Corinna Hermann and Sonja von Aulock, involving dozens of PhD and master students and many others. Once we had a cheap, reproducible test for NEP, i.e., the whole blood cytokine release assay, we could use it to optimize isolation especially of lipoteichoic acids (LTA) from Gram-positive bacteria, and to study their biological effects in comparison to LPS (Draing et al., 2008; Bunk et al., 2010).

Seventh lesson: There are products on the market that are contaminated with non-endotoxin pyrogens. The extent of this problem will only be known when MAT-like technologies are introduced for screening.

7 The importance of international harmonization

Products, especially drugs, are sold internationally, which means that only batch-release tests, e.g. pyrogen tests, that are accepted in all markets will be used. This is a killer for almost all alternative methods. The general hesitance to swap an established safety test for a new test for a given product adds to this.

Traditionally, the US is most important for pharmaceutical companies, since here 6% of the world population in 2013 consumed 41% of all drugs sold worldwide and 55% of the drugs under patent[11]. The partial acceptance by ICCVAM of the whole blood pyrogen test only as an endotoxin test had dramatic effects: From this point of view, the test is only a competitor of the cheaper and well-established LAL but not of the rabbit assay. This is not completely logical, as the LAL is considered a replacement for the rabbit test that clearly is restricted to endotoxins, while there is ample evidence that the whole blood pyrogen test covers all known human-relevant pyrogens. In 2012, the FDA issued "Guidance for Industry: Pyrogens and Endotoxin Testing: Questions and Answers"[12]. According to the ICCVAM website, "*This document is addressed to biological product, drug, and device manufacturers and is meant to clarify FDA's current position on pyrogen testing and acceptance criteria. The guidance discusses approaches such as pooling samples for testing that could reduce animal use and states that in vitro monocyte activation pyrogen tests similar to tests evaluated by ICCVAM may be used in lieu of the rabbit pyrogen test or the bacterial endotoxin test given appropriate product-specific validation*."

How did it come to this statement? The ECVAM evaluation had found sensitivities and specificities for the different variants of the whole blood assay between 85 and 99%. This must be compared to the 58% specificity and 88% sensitivity for the rabbit to detect endotoxin in saline (see above), i.e., without any possible interference of a test drug (Hoffmann et al., 2005b). Furthermore, we discussed above the poorer performance of the LAL on the very same samples. The validation study had shown that all MAT assessed correctly as negative five materials of plant and fungal origin that are false-positive in the LAL; lastly, and probably most importantly, all MAT detected as pyrogenic lipoteichoic acid from Gram-positive bacteria, the likely "Gram-positive endotoxin" (Rockel and Hartung, 2012), which the LAL fails to detect. An easy winner, one might assume.

The final verdict, however, was:

"*Based on this evaluation, ICCVAM recommends that, although none of these test methods can be considered a complete replacement for the RPT [rabbit pyrogen test] for all testing situations for the detection of Gram-negative endotoxin, they can be considered for use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis, subject to validation for each specific product to demonstrate equivalence to the RPT, in accordance with applicable U.S. Federal regulations (e.g., U.S. Food and Drug Administration [FDA]). When used in this manner, these methods should be able to reduce the number of animals needed for pyrogenicity testing. Pyrogenicity testing may involve more than slight or momentary pain or distress when a pyrogenic response occurs. Accordingly, alternative test methods must be considered prior to the use of animals for such testing, as required by U.S. Federal animal welfare regulations and policies. Therefore, these and other in vitro alternative test methods should be considered prior to the use of animals in pyrogenicity testing and should be used where determined appropriate for a specific testing situation. Use of these methods, once appropriately validated, will support improved animal welfare while ensuring the continued protection of human health. …also concluded that the currently available database does not support the use of these test methods to detect a wider range of pyrogens, as suggested in the original ECVAM submission. However, ESAC concluded that these tests "can currently be considered as full replacements for the evaluation of materials or products where the objective is to identify and evaluate pyrogenicity produced by Gram-negative endotoxins, but not for other pyrogens." ICCVAM has concluded that the current validation database for these test methods is inadequate to support such a definitive statement based on the ECVAM validation study design, which did not include biologics or medical devices and evaluated only a limited range and number of pharmaceutical products. Additionally, no RPT data were generated with the same test samples used in the in vitro test methods (i.e., parallel testing)*."

How was this possible? It was a painful learning experience for me as the then head of a validation body to see how validation peer-review takes place: ICCVAM invited 13 experts to find possible problems in the assays and their validation during a two-day meeting and in writing. I and others involved in the development and validation were present but allowed to speak only if asked. Experts included the "father" of the LAL assay ("*no conflict of interest…*") and a very vocal industry representative who viciously challenged the validation. When asked privately by the late Thomas Montag-Lessing from Paul-Ehrlich-Institute why he was so critical about the assay, he frankly said, he liked the assay but his company was concerned about new batch release tests endangering product release. During the entire process, no word was lost on the shortcomings of the assays that were to be replaced …

> ***Eighth lesson: Applying scrutiny to tests that are to be validated but not to the ones in use (the point of reference), which were never validated, and not acknowledging their shortcomings, creates hurdles for new tests where common sense would argue differently. Squeezing out of a group of experts only critical arguments about the new test and not asking them to compare it to the existing one does not do justice to new tests. Conflict of interest of experts needs to be considered more seriously.***

The overall result was a list of recommendations on what it would take to make MAT assays a full replacement (Text Box 1). We did a rough estimation of the costs of such a validation study, which came to $5-25 million, based on the more than $3 million spent on the validation studies evaluated by the pan-

[11] http://www.efpia.eu/uploads/Figures_2014_Final.pdf

[12] http://www.fda.gov/drugs/guidancecomplianceregulatoryinformation/guidances/ucm314718.htm

el. It was quite obvious that nobody would be willing to finance this and thus it was an insurmountable roadblock in the path to the implementation of the assays.

This judgment has plagued the MAT assays since. Hardly any company, if not facing serious problems of pyrogen testing with current technologies, implemented the new assays on this basis. In order to overcome this, our kit manufacturer, Biotest at the time, actually wanted to tackle this challenge and I presented our suggestions for a limited validation study to the ICCVAM pyrogen test working group and then to the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) to ICCVAM in 2011. The response was a high prioritization for such a study.

And then a miracle occurred: At an informal discussion meeting between ICCVAM, FDA and the sponsors of the suggested study during the World Conference on Alternatives and Animal Use in the Life Sciences in Montreal in 2011, ICCVAM and FDA suggested that we produce a background review document on the existing evidence that the whole blood assay detects non-endotoxin-pyrogens instead of performing a validation study. We happily agreed and submitted this a little more than a year later in December 2012 (Hasiwa et al., 2013a). Since then, we have not received any official answer. To the best of our knowledge, the issue has not been discussed since, due to new priority settings. It is difficult to understand how the use of 400,000 rabbits worldwide per year despite an existing alternative cannot be a priority. If an agency, or here a committee of several agencies, prompts certain work, I do not understand how it is possible then not to respond to it.

Ninth lesson: An incredible lack of implementation of the accepted method results in unnecessary animal use of a remarkable extent (more than 170,000 rabbits in Europe per year), because nobody feels responsible for follow-up. The only partial acceptance of MATs by ICCVAM is a roadblock.

ICCVAM Recommendations for Future MAT Validation Studies May 2008*

Future validation studies should include the following considerations:

1. Both endotoxin-spiked and non-endotoxin spiked samples should be included. Non-endotoxin pyrogen standards should be characterized prior to their use in any study, if possible.
2. All aspects of the studies should comply with Good Laboratory Practices.
3. Future studies should include products that have intrinsic pro-inflammatory properties in order to determine if these tests can be used for such substances.
4. Optimally, a study that includes three-way parallel testing, with the *in vitro* assays being compared to the RPT and the BET, should be conducted to comprehensively evaluate the relevance and comparative performance of these test methods. These studies may be conducted with historical RPT data provided that the same substances (i.e., same lot) are tested in each method. Based on ethical and scientific rationale, any *in vivo* testing should be limited to those studies that will fill existing data gaps.
5. Test substances that better represent all categories of sample types (e.g., pharmaceuticals, biologicals, and medical devices) intended for testing by the methods should be included.
6. The hazards associated with human blood products should be carefully considered, and all technical staff should be adequately trained to observe all necessary safety precautions.
7. Formal sample size calculations should be made to determine the required number of replicates needed to reject the null hypothesis at a given level of significance and power. For reliability assessments, formal hypothesis testing is essential with the alternative hypothesis being no difference between groups.

* http://ntp.niehs.nih.gov/iccvam/docs/pyrogen/tmer/exec-sum.pdf

8 The whole blood pyrogen test is an enabling technology – it does far more than replace the rabbit pyrogen test and it could do even more... (the missed opportunities)

Alternative methods are typically seen and evaluated on how well they replace the current (animal) test. However, they can often do things current assays do not. To use the example of the whole blood assay:

– Human relevance and potency: As discussed in contrast to LAL, potency and relevance of pyrogen types for humans is reflected; in contrast to the RPT the assay is quantitative, includes positive controls and broadens the spectrum of detectable pyrogens and products.
– The possibility to measure on surfaces allows determining adsorbed (filtered) samples without prior dissolution (which is very difficult for lipophilic LPS and LTA) directly on these surfaces. This enables complete measurement of airborne pyrogens (Kindinger et al., 2005), the enrichment of pyrogens for high-sensitivity measurements, e.g., for dialysis solutions (Daneshian et al., 2008), or the isolation of pyrogens from interfering, e.g., cytotoxic, samples (Daneshian et al., 2006).
– Inter-individual responses can be tested using different blood donors, which seem to play a role for some non-endotoxin pyrogens.
– Certain products that cannot be tested adequately in either LAL or RPT can now be tested by MAT, e.g., medical devices, nanoparticles, cell therapies.

9 Missed opportunity: Cell therapies and blood transfusion products

Cell therapies are an excellent example for a product type that cannot be tested by LAL or RPT. The increasingly emerging cell therapies for human use (CGPT: Cellular & Gene Thera-

py Products, in Europe: ATMP, Advanced-Therapy Medicinal Products) but also traditional blood transfusions, especially with the advent of novel pathogen inactivation approaches, have not been adequately covered yet in view of the limitations of the LAL for cell preparations. Blood transfusion products are highly regulated and normally not considered as cell therapies, but they share with CGPT the lack of established pyrogen testing; their microbiological contamination is much less likely, but the enormous frequency of their use adds up to a considerable risk – facts from the US Red Cross: "*Every two seconds someone in the U.S. needs blood; More than 41,000 blood donations are needed every day; A total of 30 million blood components are transfused each year in the U.S.*." Based on 2004 data, nearly 3 million platelets transfusions are administered in the United States per year (Palavecino et al., 2010). Approximately one in 1,000-3,000 platelet units is bacterially contaminated and poses a threat of transfusion-related sepsis, which has a 20-30% mortality rate (Brecher and Hay, 2005; States and Banks, 2005). The available data indicate that transfusion-associated sepsis develops after 1 in 25,000 platelet transfusions and 1 in 250,000 red blood cell transfusions (Blajchman et al., 2005). Based on recent studies with optimal culture methods of expired platelet-rich plasma (PRP), the prevalence of bacterial contamination is estimated to occur in approximately one in 750 to one in 1000 platelet concentrates (Corash, 2011). This would suggest 900-1125 fatalities in the US per year due to this product only. Assuming that the average hematology oncology patient may receive seven platelet concentrates during a 28-day period of support, the risk of exposure to a contaminated platelet concentrate is in the range of one in 150 per patient (Corash, 2011): "*This level of risk would not be acceptable for other intravenous medications*." Several studies (Yomtovian 2004; Yomtovian et al., 2006, 2007) identified the major sources of contamination as donor skin and asymptomatic bacteremia. Less frequently, contamination of PRP may occur during processing or manufacture of blood collection devices (Blajchman et al., 2005). "*The most common species identified in these studies were Staphylococci spp., Streptococci spp., Proprionibacterium spp. and Corynebacterium spp*." (Corash, 2011) showing the special need to cover Gram-positive bacteria and non-endotoxin pyrogens in pyrogen testing.

The advent of pathogen inactivation techniques for PRP and erythrocyte concentrates further brings up the question of pyrogenic contamination in these then sterilized products, as the sterilization procedure does not destroy the pyrogens. Noteworthy, bacterial screening and pathogen inactivation are handled quite differently in various countries (Pietersz et al., 2014).

Preparing advanced cell therapy products often requires complex procedures, such as multiple cell selection steps, *ex vivo* expansion, cell activation, encapsulation and genetic modification, which all pose a risk for microbiological and pyrogenic contamination. However, discussions about quality and safety typically do not yet include this consideration.

There is evidence that the MAT is capable of covering the possible pyrogens for cell therapies (Montag-Lessing et al., 2007; Schindler et al., 2009; Hasiwa et al., 2013a). It also covers whole bacteria as already shown in 1999 (Fennrich et al., 1999a). Noteworthy, the same number of bacteria, heat-inactivated or treated with antibiotics, led to stronger immune activation than the live bacteria, which might be of interest for pathogen inactivated products. The not yet optimized detection limit of 10^4 live bacteria is close to what is shown to be relevant by Jacobs et al., (2008): "*A detection method with a 10^3 colony-forming units/ml threshold would detect 90% of contaminants*." Notably, up to 10^9 CFU have been found in expired platelet concentrates.

Cellular therapeutics have been defined as living cells that are transferred into the intact organism in order to introduce a new function or restitute a defective one (Rosenberg, 2001). This includes a wide variety of cells such as chondrocytes, (hematopoietic) stem cells, bone marrow cells and blood cells such as activated lymphocytes, and the traditional products of erythrocytes and platelets. The latter pose a particular problem since they are stored and agitated at room temperature in bags permeable to air and are, therefore, easily subject to extensive bacterial growth if contaminated with bacteria due to endogenous infection of the donor, venipuncture or processing. Transfusion reactions ranging from rigors, fever and chills, all the way to septic shock have been described. The problem is under- rather than overrated, since numerous clinical events are not recognized as being transfusion associated but are attributed, rather, to the underlying disease.

Additionally, medication and immunosuppression of the patient might mask an existing septic/pyrogenic event. Recently, attention has focused on viral infections, although the incidence of HIV in blood products is less than 1 in 1,000,000 per unit (1 in 3,000 for bacterial contaminations) (Blajchman et al., 2005). Endotoxin determinations have been performed in some studies (Jacobs et al., 2008; Palavecino et al., 2010), but they are limited to Gram-negative infestations, which represent the minority of cases (e.g., out of 52 contaminated platelet concentrates reported by Jacobs et al., 2008, four were of Gram-negative origin), and do not allow determining cell-bound pyrogens. Two large studies in France (BACTHEM study, Perez et al., 2001) and the USA (BaCon study, Kuehnert et al., 2001) revealed that platelets hold a significantly higher risk of bacterial contamination than erythrocyte concentrates, irrespective of their being single-donor or pooled preparations. Pathogens associated with bacteremia in the US study were 59% Gram+ (mainly skin contaminants such as *Staphylococcus spp.* and *Streptococcus spp.*, and *Propionibacterium spp.* bacteria) and 41% Gram-negative (*E. coli*, *Serratia spp.*, *Enterobacter spp.*). *Yersinia enterocolitica* was not found, although it occurs frequently in transfusion-related sepsis and was responsible for 7 of the 8 recorded fatalities in the US 1986-91 as it can grow also in cold storage; this has, however, been strongly reduced by leukocyte filtration. Incidences increased with prolonged storage, and both studies linked fatalities to the occurrence of Gram-negative bacteria. The US study determined endotoxin levels as well (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. All in all, Gram-negative bacteria tended to occur more frequently in red blood cells, probably due to the storage conditions.

In July 2004, a roundtable meeting on bacterial detection, held during the Annual Congress of the International Society of Blood Transfusion in Edinburgh, discussed the limitations of current testing methods. Currently, culturing methods, such as the very sensitive BacT/ALERT method, are considered the best, though time-consuming (12 h to 7 days). Concentrates are released on a "negative-to-date" basis and recalled if necessary. The panel reported occurrences in the Netherlands, where platelet concentrates containing skin contaminants 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 (Blajchman et al., 2005). Additionally, facultative anaerobic bacteria (*Corynebacterium spp*.) are picked up later, and there are extra costs involved. However, anaerobic bacteria have been reported in fatal septic transfusion incidents (McDonald et al., 1998). Altogether, culture methods are still the method of choice but incapable of providing complete safety and other, especially faster, methods are sought. An inactivation method, the photochemical treatment (PCT) (Wollowitz, 2001), has been developed. Still, it must be kept in mind that this inactivation will inhibit growth but have no influence on the already existing pyrogenic content or might even increase it as shown above.

Therefore, the testing of these cells and their suspension materials is an interesting future challenge for the MAT. Pretesting of clinical grade erythrocytes and thrombocytes intended for transfusion indicated interference-free retrieval of an endotoxin spike (Schindler et al., 2009) when compared to the saline control. Possible masking of pyrogens binding to cells or remaining plasma proteins is a special concern for the LAL (Wakelin et al., 2006). Noteworthy, the whole blood MAT has been show to handle this especially well (Spreitzer et al., 2002; Perdomo-Morales et al., 2011).

The preliminary work on blood products has been expanded by our partners from the Paul-Ehrlich-Institute, demonstrating the detection of various UV-inactivated bacterial species in five platelet concentrates each (unpublished, personal communication by Dr Ingo Spreitzer). In 2001, we showed that intact fibroblasts do not activate the whole blood MAT and do not impair endotoxin recognition (Schneider and Hartung, 2001). First experiences of the use of MAT with cell therapy products were obtained in a collaboration with TeTec, Tübingen, Germany, in 2003 (http://www.tetec-ag.com). Their cartilage product (NOVOCART) obtained registration using MAT data (unpublished proprietary data). This would lay the basis for now systematically addressing cell therapy products. However, the respective proposal to FDA after advanced discussions was not funded in 2014.

Tenth lesson: Bacterial and pyrogen contaminations of blood products and cell therapies kill thousands of people per year (in the US alone about 1,000 people die from platelet-rich plasma). The whole blood pyrogen test seems to be a solution, but nobody exploits it.

10 Missed opportunity: Pyrogen-control of dialysis fluids

Beside kidney transplantation, dialysis is the only treatment for renal failure. The two common procedures, renal and peritoneal dialysis, employ the principle of diffusion through membranes to remove waste and excess water from the body fluid. According to European and US Pharmacopoeia standards, all substances and devices administered as parenterals must be tested for pyrogens. The RPT and the LAL still are the standard methods to test tubes, membranes, and the dialysis solutions. Still a certain percentage of patients develops fever following dialysis, sometimes traceable to pyrogens (Karanicolas et al., 1977; Mangram et al., 1998). For example in 2001-2003, a global recall was issued for a certain icodextrin-containing dialysate. Martis et al. (2005) investigated the material, which led to abdominal pain, nausea, vomiting, diarrhea, fever, and emitted a cloudy dialysate. The batches were clean in the LAL assay, and showed no temperature rise in rabbits. Using MAT they found a correlation between the incriminated batches and increased cytokine release, suggesting the presence of NEP contaminations. Later these were traced to the Gram-positive bacterium *Alicyclobacillus acidocaldarius*. Noteworthy, we showed that an adaptation of the whole blood MAT can test dialysis solutions with adequate sensitivity (Daneshian et al., 2008). However, the test is not yet applied in practice.

11 Slow implementation of the pyrogen test for medical devices

For more than 15 years we have been working with ISO TC 194 for biological testing of medical devices to implement the whole blood pyrogen test. Still, it is only mentioned, not recommended. Due to manufacturing and handling, medical devices can bear pyrogens on their surface, which, when brought into the human organism, lead to inflammatory reactions and reduce biocompatibility. Recognizing this problem, in Europe 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) in the US stated in 2001 that products with direct or indirect contact with the circulation system or the lymph, or which interact systemically with the body, should be tested for pyrogens (AAMI, 2001).

Products with direct (e.g., blood bags, needles) and indirect (e.g., swabs, gloves) contact to the blood circulation can have 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. After eight pyrogenic reactions in 69 patients undergoing heart catheterization, Kure et al. described endotoxin contamination of extracts of the hospital surgeon's latex gloves, which evoked fever in rabbits and could be successfully transmitted to cardiac catheters (Kure et

al., 1982). Grötsch et al. were able to evoke fever reactions in rabbits with an eluate of gloves containing up to 2560 endotoxin units (Grötsch et al., 1992).

However, 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 mode 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 the lipophilic pyrogens from its surface, and the dilution of such released pyrogens in a large volume of rinsing water also reduces the limit of detection. For LTA, it has been shown that surface presentation increases potency about 1,000-fold (Deininger et al., 2008), raising serious doubts about the value of eluates. The alternative of transplanting the questionable material directly into the rabbit (where this would be possible with regard to the dimensions of the material) 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 whole blood MAT 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 (Hasiwa et al., 2007). This has been demonstrated using aneurysm clips as proof of principle (Mazzotti et al., 2007). It has also recently been successfully adapted to eye lenses, etc. (Werner et al., 2009; Hasiwa et al., 2013a). Others have used the test for extracts from gelatinous polymers (Mohanan et al., 2011) and stents (Stang et al., 2014a,b).

Testing for the inflammation-inducing potential of implant surfaces for the assessment 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 where it displays manifold functions (for review see Anderson and Miller, 1984; Ziats et al., 1988). Its specific preference for rough and hydrophobic surfaces differs from that of fibroblasts (Rich and Harris, 1981). The role of cytokine production of the monocytes/macrophages in the early stages of implant insertion are, until now, poorly understood. The fact that some materials are obviously capable of modulating the cytokine response (Soskolne et al., 2002; Refai et al., 2004) 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 model was developed for the thorough depyrogenation and testing of metallic or plastic surfaces with the whole blood test in order to gain experience about possible inherent activating or inhibiting characteristics of materials (Kullmann, 2002). The study showed that pyrogenic contaminations on surfaces, generated, e.g. by them being passed from hand to hand, can be reliably removed only when heated for 5 h at 300°C. This applied to titanium, titanium alloy ($TiAl_6V_4$), and steel material used for implants. The blood was incubated directly in a depyrogenized microtiter plate with contact to the surface to be tested. Artificial contaminations were recognized in a dose-dependent manner. The MAT was also applied to develop novel pyrogen removing technologies by plasma discharge (Hasiwa et al., 2008).

Some medical devices are absorbed completely by the body, as are any contained pyrogenic contaminations. Examples are liposomes and alginate microcapsules as carriers of drugs. With the latter, the whole blood MAT could clearly identify samples as positive or negative (Schindler et al., 2009).

Pyrogenicity of nanomedicines might be considered a special form of immunotoxicity; however, it is typically not produced by the test material but by contaminations, mostly bacterial endotoxins. Nanoparticles (NP) represent a most interesting test material because of their large surface area, putatively binding these highly lipophilic endotoxins (Ashwood et al., 2007). The LAL has problems with the testing of nanoparticles. It remains to be explored whether NP qualify for testing in the MAT. This test is unique, as it works with a cell suspension, which might be especially suited to test NP (Hartung and Sabbioni, 2011).

The field of pyrogen testing of medical devices has, despite these promising preliminary results and some sporadic interest (ISO work groups, a well attended webinar for SOT, a workshop held in Baltimore 2013), not really embraced pyrogen testing. It is my impression that the main reason is that eluate testing with rabbits or LAL is almost always negative for the simple reason that endotoxins are so sticky and the molecules that are eluted are strongly diluted by the procedure. The impact of our data showing how manual handling results in pyrogen contaminations on the surface not removable with routine autoclaving or sterilization techniques has not been grasped. The paradigm shift of testing on the surface of a medical device represents an enormous opportunity to improve the safety of medical devices.

Eleventh lesson: Pyrogen testing on the surface of medical devices is a paradigm shift advancing the safety of these products, but implementation is slow. Nanomedicines might change this. A key point is that the effect of pyrogen contamination for patients has not been studied and thus hazard is somewhat hypothetical.

12 Emerging opportunity: Airborne pyrogens

Microbial cell wall components are ubiquitous in the environment. Aerosols or dust containing bacteria, fungi or their pyrogenic components, e.g., from feces in stables, in emulsions used in the steel industry to cool metals, in the filters of air conditioning systems, in organic waste collected in kitchens, etc., are found at various work places and in household air. Though a contribution of inhaled pyrogens to inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) is likely, the lack of a human-relevant quantitative measurement technology has prohibited an evaluation of this risk factor. Chronic obstructive pulmonary disease (COPD) is frequent and costly for society. According to the European Lung White Book (European Respiratory Society, 2003) clinically relevant COPD is steadily increasing affecting 4-10% of the adult population and is recognized to be the fourth

leading cause of death in the world. The total cost of COPD in Europe (direct and indirect) is estimated at about 38.5 billion € whereas asthma costs amount just to 17.7 billion €. Therefore, there is a need to better understand the aetiology and pathogenesis of the disease to enable better prevention and, in consequence, reduce the prevalence and costs of treatment. Thus, newly identified risk factors, that can be targeted, promise to make a major contribution to public health. COPD arises from the interaction of genetic factors and environmental exposure (cigarette smoke, out-door and in-door air pollutions). Cigarette smoking is considered the predominant etiological factor in chronic obstructive pulmonary disease but appears to be insufficient to completely explain epidemiology and pathogenesis, and little is yet known about other causes of the disease. A number of epidemiological studies indicate a role of occupational exposure to airborne pollutants in the initiation and progression of COPD. As many as 20% of COPD cases have been attributed to occupational air-pollutants (Trupin et al., 2003), among which gases, vapors, mineral dust and organic dusts are of main interest (Heederik et al., 2007). Especially organic dust seems to play a major role, as it affects a large number of workers, including many workers in agriculture, in recycling, in the metal industry, etc. (Gorny and Dutkiewicz, 2002). It is not clear to which extent such dust exposures represent an additional risk factor for COPD. The characteristic inflammation in asthma results from the participation and infiltration of multiple cell types from the innate and adaptive immune system. In consequence, asthmatic patients are more reactive to environmental non-biological (e.g., diesel exhaust, tobacco smoke) and biological (e.g., allergens, pyrogens) stimuli than normal individuals (Gilmour et al., 2006; Sears, 2008). A great deal of research has been conducted on evaluating chemical composition (metals, polyaromatic hydrocarbons, anions, cations, etc.) of particulate matter (PM) and associated health effects; much less research has been conducted on the biogenic components of PM (Duquenne et al., 2013).

It is known that exposure to bioaerosols results in activation of airway inflammation. These observations led to a number of studies assessing the levels of endotoxin in bioaerosols (Gorny and Dutkiewicz, 2002; Spaan et al., 2006). Based on the correlations made, the Netherlands have suggested introducing an occupational exposure limit for endotoxins. It was also reported that cigarette smoke contains high levels of endotoxin and it was postulated that this might be the component of the cigarette smoke mainly responsible for its inflammatory effects (Sebastian et al., 2006). In the case of air pollutants, studies have focused on evaluating concentrations of some pollutants and correlating them with prevalence of respiratory diseases including endotoxin (Liu, 2002; Doreswamy and Peden, 2010). Fewer studies have measured molds and glucans (Bush et al., 2006; Portnoy et al., 2008; Samadi et al., 2009; Tischer et al., 2010). Airborne pyrogens appear to contribute to organic dust toxic syndrome, chronic obstructive pulmonary disease, building-related illness, humidifier lung or Monday sickness. Epidemiological studies have shown that in occupational exposure the concentration of inhaled LPS in bio-aerosols is strongly and consistently associated with reversible airflow obstruction (Kennedy et al., 1987; Schwartz et al., 1995; Milton et al., 1996; Liebers et al., 2006, 2008). Other sources for endotoxin-containing aerosols with health effects for workers are metalworking fluids (Thorne, 2003): these lipid / water emulsions are circulated at room temperature, often for months and bacterial contamination is quite common. We have shown that these liquids, which cannot be tested by LAL, are compatible with the whole blood test (unpublished) and carried out pilot studies with respective professional association in Germany.

We adapted the whole blood assay earlier for the measurement of airborne pyrogens (Fennrich et al., 2001; Kindinger et al., 2002, 2005). The major advantages compared to LAL are that it reflects the up to 10,000fold difference of potency of different endotoxins seen in rabbit tests and can be carried out directly on a filter used for particle sampling without extraction of questionable efficiency. This application has been further evaluated by European agencies (Liebers et al., 2009, 2012; Bernasconi et al., 2010) and others (Zucker et al., 2006). The German Federal Institute for Occupational Safety and Health organized a conference on the topic in 2002 (Fennrich, 2002). We recently showed that the whole blood assay can also determine the inflammatory potential of spores from higher fungi (Rivera-Mariani et al., 2014), which are abundant in urban and rural air and associated with occupational diseases, e.g., of mushroom farmers. In addition, using subject-specific, rather than pooled, blood, the whole blood assay can also be used as an individualized immune function assay with the same stimuli. This is pioneered in our current NIH project[13].

The whole blood assay offers promising new avenues for assessing exposure response relationships in asthmatics and COPD patients and may highlight important new risk management opportunities that target biogenic components of PM. Sampling and analysis of air quality is therefore the first step to determine whether the surrounding poses a potential threat to exposed persons. The major problem in this field has been the lack of a standardized relevant methodology measuring total microbial burden, its inflammatory potency and the lack of threshold values. Despite the reality of occupational exposure to endotoxins and the acknowledgement of its effects on the health of workers, an occupational exposure limit (OEL) has not yet been established for this hazard. The whole blood system has been previously used to assess the immunoreactivity of asthmatic children to known biological pollutants (Braun-Fahrländer et al., 2002).

Current efforts extend this research into studies of occupational exposures in animal handling facilities, e.g., a PhD thesis by Linda J. Mueller-Anneling at the University of Iowa 2004[14]

[13] Hansel (PI) 3R01ES018845-04S1, May 2013 – Apr 2015, NIEHS "Genetic Susceptibility to Asthma and Indoor Air Pollution in Peru (ViCTER)"

[14] http://ir.uiowa.edu/cgi/viewcontent.cgi?article=1295&context=etd&unstamped=1

Tab. 7: A little who-is-who in the development of the whole blood pyrogen test

Name	Role
Ingeborg S. Aaberge (with L. M. Naess and B. Mogster)	Partner in validation study at NIPH, Oslo, Norway
Wilhelm Berg	Patent attorney, Germany
Susanne Berthold (with P. Zwerenz, W. Klingler and R. Dostatni)	Pyrogen project manager at DPC Biermann, Germany
Audrey Borel (with G. Schmitz)	Pyrogen project manager at Merck-Millipore, France
Rogier Bos (with M. vam Aalderen, M. Gommer and R. Nibbeling)	Partner in validation study at RIVM, Utrecht, The Netherlands
Peter Brügger (with E. Frey)	Partner in validation study at Novartis, Switzerland
Sandra Coecke (with G. Bowe, J. Casado and J. de Lange)	ECVAM, partner in validation study
Mardas Daneshian	PhD on fungal and airborne pyrogens as well as problematic test products (dialysis, cytotoxic and lipophilic drugs); joint first author background review on non-endotoxin pyrogens (Hasiwa et al., 2013a)
Stefan Fennrich	Project manager for the pyrogen test in our group for many years, continues to promote the technology from University of Tübingen, Germany
Matthias Fischer	Paul-Ehrlich-Institute; early evaluation of the test (Fischer et al., 1998)
Marlies Halder	Led the ECVAM peer-review and hand-over to ICCVAM
Thomas Hartung	Inventor and principal investigator
Nina Hasiwa	PhD on pyrogen testing of medical devices and removal from surfaces; joint first author background review on non-endotoxin pyrogens (Hasiwa et al., 2013a)
Kilian Hennes	Cryoblood validation study partner at Qualis, now Zwisler Laboratories, Konstanz, Germany
Sebastian Hoffmann	Biometry of the validation studies and modeling of rabbit responses (Hoffmann et al., 2005, 2006)
Foster Jordan (with B. Knörzer)	Kit marketing by Charles-River Endosafe (CEO)
Thomas Jungi (with M. Brcic)	Partner in validation study at University of Berne, Switzerland
Felix Rivera-Mariani (with P. Breysse and J. Negherbon)	Post-doc JHSPH on airborne pyrogens
Thomas Montag-Lessing	Early partner at the Paul-Ehrlich-Institute in the development and validation of the test
Siegfried Morath (with C. Draing, S. Deininger, R. R. Schmidt, I. Figueroa-Perez, A. Stadelmaier, A. Geyer, A. Kinsner, K. Hoebe, S. Traub, C. Hermann, S. von Aulock and others)	Developed in his PhD the isolation procedure for lipoteichoic acid, the key non-endotoxin pyrogen (Morath et al., 2001) used as reference material in the kits; showed the activity of the respective synthesized structures (Morath et al., 2002; Deininger et al., 2007) and enabled their biological characterization.
Rolando Perdomo Morales (with Z. Pardo-Ruiz)	Adaptation and evaluation in Cuba
Stephen Poole (with Y. Mistry)	Partner in validation study at NIBSC, UK and developer of one-plate version (Poole et al., 2003)
Octavio Presgrave (with I. Fernandes Delgado and others)	Adaptation and evaluation in Brazil (BraCVAM)
Wolfram Riedel	Recognized the potential of the technology and brokered the first license with DPC Bierman, Bad Nauheim, Germany
Stefanie Schindler	PhD and DVM/PhD thesis on the test including optimization of cryopreserved blood, validation studies, testing of lipophilic samples and airborne pyrogens.
Anke Schulz (with B. Holtkamp and G. Schmitz)	Pyrogen project manager at Biotest, Germany
Ingo Spreitzer (with B. Loeschner, S. Müller and others)	PhD on non-endotoxin pyrogens; then work on pyrogenicity testing at the Paul-Ehrlich-Institute
Sonja von Aulock	Studied the variance of whole blood responses in large donor collectives (von Aulock et al., 2003, 2004).
Markus Weigandt, (together with M. Jahnke, P. Lexa and H.-G. Sonntag)	First independent evaluation of the test as thesis prompted by European Pharmacopoeia and financed by Roche-Boehringer Mannheim (Weigandt et al., 1998; Jahnke et al., 2000)
Albrecht Wendel	Co-inventor providing the infrastructure for R&D and partner for the commercialization
Gabriele Werner-Felmayer (with A. Peterbauer and P. Loitzl)	Partner in validation study at Innsbruck University, Austria

(Mueller-Anneling et al., 2006) and by Jesse Negherbon at Johns Hopkins (unpublished) and might be extended to studies of sick building syndrome, and studies of elderly persons suffering from pulmonary disease (e.g., COPD).

Twelfth lesson: Due to the lack of a human-relevant measure of airborne pyrogens up to now, we are only starting to learn how much this may be contributing to the ever increasing incidence of obstructive lung diseases in children and adults.

13 Acknowledging the team

An enormous number of colleagues, technicians, students and others have been involved over these twenty years. It is impossible to acknowledge them all. Probably, I forgot to mention quite a few. Table 7 lists some of the major partners. I am most grateful for their support.

14 Conclusions

These 20 years have been a fascinating journey. Working with the prime pyrogen, endotoxin (LPS), and its inflammatory effects, I was still not aware of the challenges of pyrogen testing when this all started. The vigorous response of our immune system is the only way to live with ten times more bacteria in and on our body than own human cells. I often use the following calculation to illustrate this: Our gut contains 1-2 kg of bacteria with about 50 g of endotoxin; if isolated and injected into humans, this could kill one million people or induce fever in one billion.

Our body's reaction to pyrogens has to be extremely fine-tuned: too little response and we suffer from infection, too much and we suffer from inflammation. This is probably the reason for the extraordinarily preserved reaction between individuals: With hundreds of donors we have not seen major deviations as to the threshold concentrations of LPS they react to and the cytokines released.

The whole blood test represents the simplest human cell culture imaginable, especially when using cryopreserved blood: go to the fridge, thaw blood in a thermoblock, add cell culture medium and the test sample, incubate, shake, take a sample for cytokine detection. For the latter we even experimented with dip-stick solutions with DPC Biermann, which worked well but were unfortunately never marketed.

We have so far found no product that could not be tested for pyrogens with a variant of the test. This includes many products that are problematic in LAL or rabbit tests, such as cell therapies, solid materials or cytotoxic substances.

The most incredible experience, however, was to see how slow, if at all, the implementation of an alternative method is, even when it is accepted by authorities. We totally lack the means for enforcement, which in our case leads to the unnecessary sacrifice of hundreds of thousands of rabbits per year. I consider this a scandal.

The lack of implementation also kills people: The non-endotoxin pyrogen safety gap is a reality. The lack of control of blood transfusions for bacterial and pyrogenic contaminations is incomprehensible – here we know that hundreds to thousands of people die every year, and while we do everything to prevent HIV transmission no action is taken to implement a promising solution to stop bacterial or pyrogen transmission.

If you have a hammer, everything looks like a nail… Over the years we found many applications of the pyrogen test for more and more product groups, for immunotoxicity tests (Langezaal et al., 2002; Hartung and Corsini, 2013), but perhaps most importantly for airborne pyrogens. Today's intense farming of animals in confined spaces means that both animals and humans inhale enormous amounts of pyrogens. This leads to work-related lung diseases and to animal health problems. I was once told that pigs are slaughtered typically around only 120 days of life and more than 60% show signs of chronic obstructive lung disease… This is certainly also an economic factor impacting on the efficiency of meat production, if the human and animal health considerations are insufficient to prompt action. At this moment we lack the epidemiological studies showing the health effects of airborne pyrogens measured with a human-relevant method. If this causality can be established, the need for pyrogen tests in controlling work and living environments will increase dramatically. Given that one blood donation of 500 ml is sufficient for more than 50,000 tests, the blood will not be the limiting factor….

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Conflict of interest statement

The author holds patents as inventor of the whole blood pyrogen test and the use of cryopreserved blood, which are licensed to Merck-Millipore; he receives royalties from Merck-Millipore from sales of the kit version.

Acknowledgment

I would like to thank Albrecht Wendel for critically reading this manuscript.

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