



“Humanity has but three great enemies: fever, famine, and war; of these by far the greatest, by far the most terrible, is fever.”

William Osler (1849-1919, Johns Hopkins University)
(Osler, 1896)

“When you solve a problem, you ought to thank God and go on to the next one.”

Dean Rusk
(1909-1994, American Secretary of State)

Food for Thought ...

Pyrogen Testing Revisited on Occasion of the 25th Anniversary of the Whole Blood Monocyte Activation Test

Thomas Hartung^{1,2}

¹Johns Hopkins Bloomberg School of Public Health, Center for Alternatives to Animal Testing, Baltimore, MD, USA; ²Center for Alternatives to Animal Testing, CAAT-Europe, University of Konstanz, Konstanz, Germany

Abstract

The whole blood pyrogen test invented 25 years ago, and its variant based on cryo-preserved blood one year later, brought momentum into the field of pyrogen testing, which, despite the broad application of the *Limulus* amoebocyte lysate (LAL) assay, aka bacterial endotoxin test (BET), consumed several hundred thousand rabbits per year world-wide. The resulting international validation and lengthy acceptance and implementation process of what are called now monocyte activation tests (MATs) finally is impacting on animal numbers – at least in Europe – reducing them by more than 70% and counting. The author sees no reason for continuing any regulatory rabbit testing for pyrogens except the lack of acceptance of MATs in some regions of the world. The availability of MATs has opened also the discussion about the shortcomings of LAL/BET, namely its restriction to Gram-negative pyrogens, non-reflection of the potency of these in humans, interference and masking by many products, and animal welfare concerns for horseshoe crabs. The obvious advantages of MATs in all these respects should lead to a shift from LAL/BET to MATs. We are starting to see this for vaccines and medical devices, but other areas like safety testing of blood transfusions, cell therapies and nanomaterials, and the assessment of air-borne pyrogens still need to grasp the opportunity provided by MATs. While the different MATs can jointly serve these needs, the whole blood MAT has some advantages as discussed here.

1 Introduction

Twenty-five years ago, Albrecht Wendel and I (Hartung and Wendel, 1995, 1996) suggested a whole blood pyrogen test in this journal. It came as a fresh breeze where several previous attempts to create a human cell-based test had somehow stalled and not made it beyond half a dozen articles. The major transition from the rab-

bit pyrogen test to the *Limulus* test, more precisely *Limulus* amoebocyte lysate (LAL) test, aka bacterial endotoxin test (BET), had seemingly exhausted the regulatory and regulated community, and they had little motivation to start something new.

Today, assays based on different preparations of human monocytes for pyrogen detection are collectively called monocyte activation tests (MATs). Our whole blood assay, and its cryoblood

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Correspondence: Thomas Hartung, MD PhD
Center for Alternatives to Animal Testing
Johns Hopkins Bloomberg School of Public Health
615 North Wolfe Street
W7032, Baltimore, MD 21205, USA
(thartun1@jhu.edu)

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variant, are two such MATs. I have summarized the lessons learned from the first twenty years in this field (Hartung, 2015) in this series, and it is not my intention to repeat them here. Some other recent reviews are also available (Fennrich et al., 2016; Spreitzer, 2019). The goal of this article, written on the 25-year anniversary of the whole blood pyrogen test, which coincides with the end of the last patent (January 10, 2021) held by the author in this field, is to give an update and share some insights of the last five years.

Most remarkably, the number of animals used for rabbit pyrogen testing is finally going down. I often called the whole blood pyrogen test my “claim to fame” for alternatives to animal tests. But, considering that now there is a group of MAT assays that includes ours, I have to refine that my major accomplishment was not the moment of inspiration 25 years ago but pushing ahead the validation and implementation of MATs in general in the time since then. It was not an easy decision to promote and push all potential human pyrogen tests I was aware of at the time together instead of focusing on our own. This was certainly owed to my early involvement with the European Centre for the Validation of Alternative Methods (ECVAM). I organized a workshop for ECVAM in January 2000 (Hartung et al., 2001) that developed the blueprint for the initial validation study with EU funding (Hoffmann et al., 2005; Schindler et al., 2006a). So, it is with pride that I now see the MATs finally extinguishing the use of rabbits for pyrogen testing, even though I had hoped to see this much sooner and with a larger contribution of our own assay. I will discuss animal numbers in more detail below, but just to give an indication of the level of achievement: At the time of our validation study, rabbit use for pyrogen testing exceeded the number of all animals used for industrial chemicals or pesticides in the EU. Between 2008 and 2018, rabbit use for pyrogen testing in Europe fell by almost 80%!

2 Does the whole blood assay have an edge over the competition?

I believe so, but this is my baby... Here is why I think it does (Fig. 1): What are the arguments and what has changed since 2015 (Schindler et al., 2009; Hasiwa et al., 2013; Hartung, 2015; Fennrich et al., 2016)? First, what are the advantages of MATs over traditional pyrogen tests such as the rabbit test and the BET?

MAT vs rabbit test

Advantages MAT: no animal use, no species difference rabbit vs. human, less labor and lower costs (less than 10%), quantitative, lower test sample volume needed (important for costly medicines like Factor VIII preparations or immunoglobulins), MAT variants can be used for chemotherapeutics and other products that cannot be tested in rabbits. The rabbit test is not applicable to solid materials, has no concurrent positive and negative controls, was never validated, and is less sensitive than humans (about 10-fold) = higher detection limit (especially for non-endotoxin pyrogens).

Advantages rabbit test: historical data, broad regulatory acceptance.

No change since 2015.

Simply said, in 25 years of work in the pyrogen field, I have not seen any product or pyrogen that could not be addressed with a variant of the MAT, but I have learned a lot about the shortcomings of the rabbit test. It is a scandal that despite clear legal obligations (at least in Europe) the practical availability of an accepted alternative has not yet led to the full replacement of the rabbit pyrogen test. But, finally, things are moving in the right direction as we will discuss.

Some new studies have compared MAT variants and the rabbit test. A direct comparison of the rabbit test and the whole blood cryo-MAT for Gram-positive lipoteichoic acid, a non-endotoxin pyrogen (NEP), demonstrated the higher sensitivity of the MAT (Gimenes et al., 2015); however, it should be noted that in that study a commercial lipoteichoic acid was used that is prepared by inadequate purification methods and is notorious for endotoxin contamination (Morath et al., 2002). Rabbits are known to be less sensitive to both endotoxins and lipoteichoic acids (Schindler et al., 2003, 2009; Hasiwa et al., 2013) as shown again in this study.

An interesting and thoroughly studied case of false-positive rabbit tests was recently reported by Zervos et al. (2019): After a number of multi-donor immunoglobulin (IgG) preparations failed the rabbit assay, this was traced back to a single donor. The febrile response elicited in rabbits by IgG was shown to be a rabbit-specific phenomenon caused by reactivity of IgG with rabbit leukocytes, while the MAT did not show any reaction. The authors concluded “*LAL [Limulus Amebocyte Lysate] and MAT, may be more appropriate than the rabbit pyrogen test, and ultimately prevent the unnecessary rejection of acceptable product*”. This shows that not only patient safety but economic consequences for producers are at stake when using the animal test.

MAT vs BET

Advantages MAT: No animal use (8-15% of horseshoe crabs die after being bled), no species differences (even stronger than rabbit test). BET is restricted to endotoxin from Gram-negative bacteria, shows false-positive reaction with glucans like cellulose and many herbal preparations, is disturbed by many products, e.g., albumin and many other proteins, vaccines, nanomaterials, human pyrogen potency is not reflected (Dehus et al., 2006), BET is not applicable to solid materials.

Advantages BET: costs and time for assay, historical data regulatory acceptance.

New since 2015: increased awareness of masking effects in BET, slow uptake of recombinant BET.

As the BET became the standard test in the 1990's, the drive to spell out its shortcomings was low. The fact that it is blind to NEPs from Gram-positive bacteria (which include the typical spore-forming bacteria likely to contaminate products) should have anyone who is concerned about patient safety sing halleluiah about the availability of MATs that close these safety gaps. (The term NEPs distinguishes this group of substances from endotoxin, known to be lipopolysaccharide (LPS) from Gram-negative bacteria for almost 70 years now.) Sure, the BET is cheaper and a bit faster, but does this matter where patient safety is at stake? What

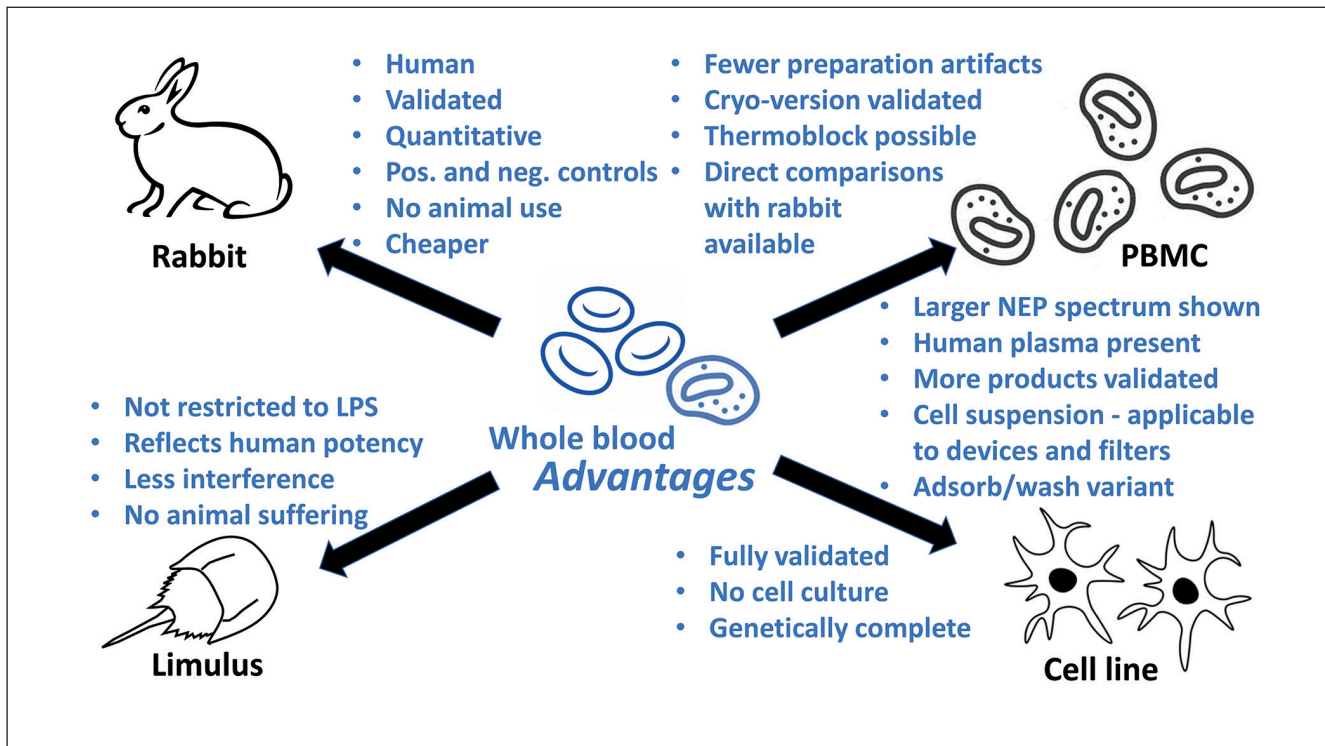


Fig. 1: Advantages of the whole blood MAT over other pyrogen tests

Source of images: horseshoe crab, rabbit: free clip art; cells: Léa Lortal, <https://thenounproject.com/term/dendritic-cell/2310037/>; Leukocyte: https://www.iconfinder.com/icons/4934332/blood_cells_hematology_platelet_test_icon; Erythrocytes: <https://icon-library.com/icon/cells-icon-10.html>

is really needed now is someone stepping up to produce a NEP reference material that is negative in BET. This could illustrate the problem of only detecting endotoxin where there is no reason to assume that contaminants from other microbiological classes are not important for product safety.

The other shortcoming of the BET that has emerged more recently is the phenomenon of *low endotoxin recovery (LER)*, which is increasingly discussed (Schwarz et al., 2017; Reich et al., 2016, 2018, 2019; Ørving et al., 2020). LER describes the inability of the BET to detect endotoxin added to undiluted drug samples, also called masking of endotoxin. This is not really a new insight but was formerly known mostly to practitioners. It used to be overcome by only spiking samples with endotoxin just before the BET to achieve good endotoxin spike recovery. In our validation study (Hoffmann et al., 2005), we reflected real-life contaminations by spiking ten different drugs with different levels of reference endotoxins and then sending them to the participating laboratories to be tested. This was no problem for the MATs, but two of the national pyrogen reference laboratories also measured them in the BET with disastrous results (Hartung, 2015). Thus, the BET is blind to such pyrogen contaminations, although they can activate the human immune system. This should ring a lot of alarm bells.

Binding of endotoxin to Factor C of the Limulus endotoxin-induced clotting cascade is the key rate-limiting step of the BET as-

say. A recombinant form of the protein (rFC) should now make the need for horseshoe crab hemolymph obsolete. Such rFC assays are currently available from Lonza (PyroGene™) and Hyglos (EndoZyme® and EndoLISA®), with similar hands-on time and sensitivity of endotoxin detection ranging up from 0.005 EU/mL (Ding and Ho, 2010; Grallert et al., 2011). Although they overcome the animal use of the BET, some standardization issues as well as the false-positive reaction of the BET to glycans, they otherwise share most limitations with the original BET. Thus, they dress up the BET without changing its inferiority as a safety test compared to the MATs. Some further limitations for special product groups were analyzed recently (Spoladore et al., submitted).

But what is the advantage of whole blood MATs over other MATs?

Whole blood MATs vs other MATs

Advantages whole blood MATs: fewer preparation artefacts (than PBMC-based MATs), autologous human plasma present, only validated cryo-variant, only MAT shown to reflect potency of different endotoxins correlating with rabbits (Fennrich et al., 1999a), MAT cell suspension allows solid material contact (e.g., after adsorption to remove interference or increase sensitivity, for medical device testing on surfaces), different donors or pooled blood can be used and genetic instability is no issue (in contrast to cell line-based MATs), lit-



tle inter-individual donor difference (but reflects donor differences in contrast to cell line-based MATs), rabbit whole blood assay described to assess species differences (Schindler et al., 2003), no need for continuous culture of cell lines or several weeks cell culture from frozen aliquots to run an assay (exception ready-to-use frozen cells).

Disadvantages whole blood vs. some other MATs: primary cell use requires donor screening for pathogens for biosafety (same as PBMC); short window of usability of freshly drawn blood if not cryopreserved (same as PBMC).

New since 2015: whole blood MATs now out of patent, other MATs also available as commercial kits, advantage of human plasma over fetal bovine serum (FBS) typically used for PBMC and cell line-based MATs shown (Molenaar-de Backer et al., 2021).

This list might be too condensed for many (see Hartung, 2015 and below for more details).

Up to 2015, the whole blood MAT was the only variant available in kit form, which represents an enormous advantage with respect to standardization and availability. Since then, a number of MAT kits have been marketed, showing the growing interest in MATs:

- Merck (MilliporeSigma)¹ – PyroDetect (Cryoblood), i.e., our assay
- Merck (MilliporeSigma)² – PyroMAT (MonoMac-6 cells)
- Solvias³ – identical to the Merck PyroMat

- MAT BioTech⁴ – CTL-MAT (cryo-PBMC)
- MAT Research⁵ – MAT research kit (cryo-PBMC)
- HaemoChrom Diagnostica⁶ – HaemoMAT (PBMC)
- Sanquin⁷ – PyroCell MAT System (cryo-PBMC)
- Lonza⁸ – PyroCell (same kit as Sanquin but distributed by Lonza; partnership for distribution announced in 2020).
- Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum – whole blood in MAT applied for Indian patent⁹ and announced¹⁰ kit development. It is not clear how this assay differs from the Merck one.

The availability of cryopreserved blood leukocytes is a key advantage for feasibility (no need for blood donors at time of assay), standardization (pre-testing for possible abnormal pyrogen reactions of donors), and biosafety (pretesting of donors for relevant human pathogens). Already at the time of the cryo-whole blood MAT validation (Schindler et al., 2006a), a cryo-PBMC variant was available, but its ring trial failed for reasons not attributed to the assay in principle, and, in consensus, this part of the study was not included in the publication. In the meantime, the cryo-PBMC approach has been published (Koryakina et al., 2014; Solati et al., 2015), but has not undergone formal validation in ring trials outside of the developing laboratories.

Table 1 makes a comparison as to the validation status of the MATs. Since 2015, the main new contribution as to validation of the cryopreserved whole blood assay was a ring trial in China among three pyrogen control laboratories (He et al., 2018), which confirmed the feasibility of the approach, essentially repeating the earlier validation trial (Schindler et al., 2006a) but with some dif-

Tab. 1: Validation status of different MAT variants

Compilation to the best knowledge of the author.

Test	Pre-validation transferability	Pre-validation non-LPS pyrogens	Validation for LPS in products	Validation against rabbit test in products
PBMC (IL-1, IL-6)	Yes	Yes	Yes	?
Cryo-PBMC (IL-6)	Missing	Missing	Unpublished	Missing
Whole blood	Yes	Yes	Yes	Yes for albumin, Factor VIII, IgG
Cryo-whole blood	Yes	Yes	Yes	?
THP-1 (2 models)	Yes	Yes	Failed	Missing
MonoMac-6	Failed	Failed	Yes	Missing
Others (e.g., TLR-transfected)	Missing	Missing	Missing	Missing

¹ <https://www.sigmaaldrich.com/technical-documents/articles/microbiology/monocyte-activation-test-for-pyrogen-detection.html2>

² https://www.emdmillipore.com/US/en/products/industrial-microbiology/pyrogen-testing/monocyte-activation-test/2cKb.qB.zawAAAE_vQR3.Lxj.nav

³ <https://www.solvias.com/news-events/2019/10/mat-a-validated-in-vitro-assay-for-detection-of-all-pyrogens.php>

⁴ <https://www.mat-biotech.com>

⁵ <http://www.matresearch.com>

⁶ http://www.haemochrom.de/fileadmin/user_upload/HaemoMAT_short_info_ENG.MELI.pdf

⁷ <https://www.sanquin.org/products-and-services/monocyte-activation-test/index>

⁸ https://bioscience.lonza.com/lonza_bs/US/en/Endotoxin-Detection/p/000000000000249735/PyroCell-Monocyte-Activation-Test-%28MAT%29-Kit

⁹ Patent India: Development of an "In vitro pyrogen test kit" for the evaluation of pyrogenicity using human whole blood, By Mohanan PV, Siddharth Banerjee, Muralidharan CV, Lissy K Krishnan and Bhuvaneshwar GS (applied); <https://sctimst.ac.in/About%20SCTIMST/Departments%20and%20Divisions/BioMedical%20Technology%20Wing/Division%20of%20Toxicology/>

¹⁰ Dr P. V. Mohanan at the International webinar & 3rd National Conference of the Society for Alternatives to Animal Experiments (NCSAAE & IWSAAE-2020) December 28, 2020

ferent pharmaceutical products, i.e., immunoglobulins, hepatitis B vaccine, pegIFN α 2b, albumin, and rabies vaccine.

In short, only the PBMC (fresh blood donation) and the whole blood assay (fresh and cryo-blood) have been fully validated for endotoxin and (blinded though not in a ring trial) for NEPs. There is little doubt that in principle cryo-PBMC would perform similarly, but ring trials of these are outstanding. Based on our own experience with the challenges around producing cryo-blood and maintaining the cooling chain when distributing it to other laboratories, validity should, however, not be taken as a given.

With respect to cell lines in general, there is considerable doubt as to whether these are equivalent to the other MATs, as only MonoMac-6 cells were partially validated, e.g., not for NEP detection. Noteworthy, two THP-1 cell line assays failed validation (Hoffmann et al., 2005). Having worked with such cell lines in my group for more than a decade, my personal experience is in line with cell lines sometimes performing excellently and in the next experiment failing completely. Certainly, there are ways of standardizing towards ready-to-use cells, e.g., by freezing. However, already the cell bank-deposited MonoMac-6 cells show genetic variability¹¹, which would require confirmation of the presence of key elements of the machinery of pyrogen detection and their expression into mRNA and proteins. Our own experience with other cell lines like MCF-7 (Kleensang et al., 2015) and that of others for HeLa cells (Fratini et al., 2015) is alarming. We also showed earlier how much this can be further disturbed by transfection (Hartung, 2013, Figure 4 there), raising additional concerns for the novel TLR-transfected MAT variants. While cell line-based assays have some advantages over primary cells with respect to biosafety, the genetic instability and use of FBS are key issues. For some unknown NEPs, for example from fungal spores, we cannot even comprehensively test whether the detection machinery is present, and all these verifications would need to be repeated in principle with every batch of cells produced. It is also difficult to understand why several donors are required for primary cells but not for cell lines, which only represent a single donor. In comparison, the blood leukocyte response from different donors is remarkably stable (von Aulock et al., 2006).

Technically, it would be relatively straight-forward to run a catch-up validation for any new MAT such as the cell line-based MATs, but it would cost about \$500,000 per assay and at least two years of work. It is not clear to me why this should not be required for assays that are considerably different from the validated ones.

The discovery of LPS (Westphal et al., 1952) as the Gram-negative endotoxin has shaped our view on pyrogens and innate immunity. The search “LPS OR lipopolysaccharide OR endotoxin” results in 160,000 hits in PubMed, illustrating the enormous research in this area. While Gram-positive bacteria (and to some extent fungi) induce similar inflammatory reactions and fever in humans, there are still no similarly well-established NEP. BET-neg-

ative products that have caused fever in rabbits and/or patients are known (Hasiwa et al., 2013); more recently, a few examples were published such as albumin batches that were positive in rabbit and MAT but not in BET (Solati et al., 2015), and a highly purified recombinant protein from Pfizer, which passed traditional endotoxin / pyrogen tests but resulted in adverse reactions in patients (one fatal) and tested positive in a TLR-transfected cell line MAT, helping to identify flagellin as the culprit (Huang et al., 2009). These examples demonstrate the clinical relevance of NEP.

In 2013, we summarized the enormous body of evidence that the whole blood MAT detects a broad variety of NEP (Hasiwa et al., 2013). Notably, there is no such broad database for other MATs. This comprehensive review was prepared upon request of ICCVAM/NICEATM, the US validation body, after discussions with the FDA pyrogen working group in 2011. I continue to repeat on any suitable occasion that our submission of this data in late 2012 never received an answer – and do so again here. I hope at least to embarrass those involved at the time, as very substantial work was spent on this. We had suggested to set up a validation management group and design the validation study to identify a number of test materials and non-endotoxin pyrogens. We asked to clarify the need for Good Laboratory Practice (GLP) and suggested LTA and lysates of Gram-positive bacteria, and rabbit testing only for spike materials. We even had some possible sponsoring for the study at hand. To our surprise, the FDA/NICEATM group we met suggested to produce the background review document instead of doing the study. In consequence, the discussion on whether our and other MATs detect non-endotoxin pyrogens (NEP) has still not been fully settled by a validation study. Dr Ingo Spreitzer of the German Paul-Ehrlich-Institute, the national control authority for sera and vaccines, nicely stated on this topic in 2019: “*Food for thought: We still discuss the MAT-risk of NEP-nonresponders while the BET-risk for NEP is 100%*”¹²

The detection of NEP – a key advantage over the BET and the basis for the full replacement of the rabbit test – should be a guiding factor for the selection of which MAT variant to use as discussed above. The whole blood MAT not only has the largest body of evidence for NEP detection on record, it also has the key advantage of having human autologous plasma present, while the PBMC-based MAT uses mostly FBS. Little is known about species differences (bovine vs human) and compatibilities for serum factors involved in endotoxin and NEP detection by the immune system. Most recently, an article was published in this journal that clearly shows that the PBMC MAT strongly benefits from using human serum for the detection of NEP (Molenaar-de Backer et al., 2021). Obviously, this is exactly the situation of the whole blood assay. The suggested human serum PBMC MAT means that human serum is first removed and then added back (from a different donor) with all associated labor and resulting need for quality assurance.

¹¹ <https://www.dsmz.de/collection/catalogue/details/culture/ACC-124>; Cytogenetics: human flat-moded hypotetraploid karyotype with near-diploid (8%) and polyloid (17%) sidelines - 84-90<4n>XX/XXX, -Y, +6, +7, -12, -13, -13, -16, -16, +2mar, t(9;11)(p22;q23)x2, add(10)(p11)x2, add(12)(q21), del(13)(q13q14)der(13)t(13;14)(p11;q12)x2, der(17)t(13;17)(q21;p11)x2

¹² Dr Ingo Spreitzer at “Monocyte Activation Test (MAT) – Hands-on Laboratory Training Course”, 28.2.-1.3.2019 in Bernried, Germany


Tab. 2: Comparison of the whole blood MAT to other MATs

	Whole blood MAT IL-1	Other MATs
Comparison to BET	More human-relevant results, less disturbance by LPS-binding, no false-positive reaction to glucans, coverage of NEP But: more costly, more time-demanding, less broadly accepted	
Validation status	Fresh whole blood and cryo-variant in ring trials (Hoffmann et al., 2005; Schindler et al., 2006a)	Only fresh blood PBMC fully validated (Hoffmann et al., 2005)
Limit of detection	Most sensitive MAT at 0.1 pg/mL LPS (0.001 EU/mL) for adsorb/wash protocol (Daneshian et al., 2008)	All MAT capable of detecting the human fever threshold; PBMC slightly more sensitive than others
NEP coverage	Broadest body of evidence (Hasiwa et al., 2013)	Lack of human serum in standard (validated) assays leads to questionable results (Molenaar-de Backer et al., 2021); no compiled database, NEP evidence often without exclusion of endotoxin contamination
Interference by test materials	No interference by aluminum hydroxide in vaccines (Carlin and Viitanen, 2005), lipidic parenterals (Schindler et al., 2006b), 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)	To the best of my knowledge no such data available
Incubation	Incubation in thermo-block possible easing QC	Incubator required
Protocol differences (Daneshian et al., 2009)	Preparation artifacts for PBMC preparation and washing after thawing avoided; no cell culture; variants for different applications available	Typically require culture for cell line-based MAT or for PBMC isolation or washing after PBMC thawing; isolated PBMC typically attach to culture plastic making direct contact incubation with medical devices or filters difficult
Medical devices	Testing on surface demonstrated (Hasiwa et al., 2007; Mazzotti et al., 2007)	---
Air-borne pyrogens	Testing on filters demonstrated (Kindinger et al., 2002, 2005; Hartung, 2015)	---
Blood transfusion and cellular therapies	Feasibility demonstrated (Fennrich et al., 1999b; Hartung, 2015)	---

BET, bacterial endotoxin test, aka Limulus ameobocyte lysate assay; LPS, lipopolysaccharide, endotoxin; MAT, monocyte activation test; NEP, non-endotoxin pyrogen; PBMC, peripheral blood mononuclear cells; QC, quality control

A number of human serum components have been identified that contribute to NEP detection, especially the LTA of Gram-positive bacteria, the substance class with most characteristics of a Gram-positive endotoxin (Rockel and Hartung, 2012):

- Soluble CD14 (Hermann et al., 2002)
- Lipopolysaccharide binding protein (LBP) (Fan et al., 1999; Hermann et al., 2002; Schröder et al., 2003)
- L-ficolin (Lynch et al., 2004)
- LDL through apolipoprotein B100 (inhibitory) (Sigel et al., 2012)
- Immunoglobulins (Bunk et al., 2010)
- Soluble CD36 (Jimenez-Dalmaroni et al., 2009)
- Mannose-binding lectin (MBL) (Ip et al., 2008)

A point of discussion has been from early on, which cytokine released by activated monocytes should be measured as a suitable MAT endpoint? The author has not seen convincing data that

there is any type of pyrogen that consistently induces the release of some but not other mediators. My personal preference has always been to measure the factor that is physiologically linked to fever induction, i.e., IL-1 β . PGE₂ is linked to fever too, but in the physiological chain of events it is produced in the hypothalamus, not the monocyte, and a small molecule like PGE₂ is also more of a challenge to measure. Others have suggested IL-6, TNF and neopterin. I do not think this really matters too much. The activated monocyte releases hundreds of mediators, and there does not appear to be much selectivity in the early ones although factors released later can be influenced by feed-back loops. In choosing the mediator to be measured as an endpoint, aspects like the amount formed, the kinetics, and the quality of the detection system available (today typically ELISA or PCR) matters most. TNF release by monocytes is suppressed more in the presence of DMSO than the release of other cytokines, which is a prob-

lem for the cryo-variants. Some mediators like IL-10 (not really suitable as its release is late) or IL-8 have additional problems of either spontaneous release by some blood donors or higher inter-individual variability.

There is also an ongoing comparison of the limit of detection for endotoxin of different MAT. Humans are about 10 times more sensitive to lipopolysaccharide than rabbits, which is compensated by the typically ten times higher injection volume in the rabbit assay. All MATs are more sensitive than the human fever threshold, again because of the large sample volume, i.e., 8-20% of the incubation. The PBMC MAT is a bit more sensitive than the whole blood MATs, likely because of the enriched number of monocytes. Furthermore, the presence or absence of human serum impacts here too, as described above (what the absence of human serum gains for LPS detection, it loses for NEP). In practical terms, these differences hardly matter, as they can be adjusted to needs, e.g., more sample and/or more blood. Noteworthy, the record of the lowest detection limit of any pyrogen test so far is to the best of my knowledge still with a variant of the whole blood MAT using albumin-coated microspheres at 0.1 pg/mL LPS (= 0.001 EU/mL) in the sample, which was developed to make low-level endotoxin contaminations in dialysis fluids measurable as patients are exposed to very large volumes of these (Daneshian et al., 2008).

Stoppelkamp et al., (2017) published an interesting paper aiming to accelerate the MAT: They found first that MATs based on primary cells were more sensitive than a cell line-based variant; quantitative real-time PCR showed IL-6 mRNA transcripts with greater changes in Ct-values (cycle threshold, i.e., the number of replication cycles to a positive signal in PCR) upon LPS-stimulation compared to IL-1 β and TNF- α , but quantification was unreliable; IL-6 protein secretion from whole blood or peripheral blood mononuclear cells (PBMC) was also best suited for an accelerated assay, with a larger linear range and higher signal-to-noise ratios upon LPS stimulation. An increase of the incubation temperature to 40°C increased not only responses to lipopolysaccharides (LPS) but also to other pyrogens by up to 13-fold. Interestingly, this is not the case for IL-1, where release decreases above 38°C (Boneberg and Hartung, 2003).

In summary, the author still sees a number of advantages for the whole blood MAT over other variants (Tab. 2), but, most importantly, all MATs do the trick for most applications.

3 Vaccines

As recounted in the 2015 article (Hartung, 2015), the idea for the whole blood MAT was sparked by a workshop on alternatives to animal testing in the fall of 1994 held at the Paul-Ehrlich-Institute, Germany. Two of the main reasons that vaccines could not transition from rabbit pyrogen testing to BET was that some intrinsically include pyrogens, both endotoxin and NEP, and that some common vaccine additives like aluminum hydroxide interfere with BET. We discussed the NEP issue above, but, importantly, alu-

minum hydroxide does not interfere with the whole blood MAT (Carlin and Viitanen, 2005). So, it is with deep satisfaction that the author sees increasing uptake of the MAT for this product type:

- *Neisseria meningitidis* vaccine (Stoddard et al., 2010)
- Hyperimmune sera (da Silva et al., 2015; Silva et al., 2018; Utescher et al., 2018)
- Meningococcal vaccines (Vipond et al., 2016, 2019; Valentini et al., 2018; Studholme et al., 2019)
- Tick-borne encephalitis virus vaccine (Etna et al., 2020)
- Yellow fever vaccine (de Mattos et al., 2018)
- *Shigella sonnei* vaccine (Gerke et al., 2015)
- Rabies vaccine (He et al., 2018)
- Hepatitis B vaccine (He et al., 2018)

Noteworthy, the most comprehensive evaluation of MATs for vaccines included nine different laboratories from nine countries covering 15 test systems for three batches of Bexsero from GlaxoSmithKline (GSK) (Studholme et al., 2019). The results of this collaborative study demonstrated a good level of accuracy for most test systems, with 13 of 15 showing greater than 80% accuracy: “Overall, the consistency and accuracy of the MAT was remarkable given the range of test systems used by participants, all of which are permitted by the Ph. Eur. General MAT Chapter”.

Rossi et al. (2020) discuss the potential of MATs for vaccines based on outer membrane vesicles: “MAT has the potential not only to assess the absolute difference in comparison to the same compound and batch-to-batch variation but also to assess the relative difference between compounds produced with different strategies (i.e. by different genetic modification of lipid A). The latter aspect is useful for screening different vaccine candidates during discovery, ... as it does not require animal experimentation (that is more expensive and less ethical).”

This shows applicability for a number of (new) vaccines. The pressure should be mounted on producers of established vaccines to transition to MAT – the principal onus here is with the different pharmacopoeias, and – at least in Europe – this is a legal requirement. For example, approximately 5,850 influenza vaccine batches were tested by the European Pharmacopoeia OM-CL network between 2006 and 2016 (Kretzschmar et al., 2018), a product that requires pyrogen testing. This shows the extent of pyrogen testing necessary for vaccines. “Vaccine preparations, including those for virus-mediated diseases such as COVID-19, are routinely tested for pyrogenic contaminants before distribution to the public.”¹³ So we can imagine the current boost for pyrogen testing. It is important that this does not take place in rabbits and better not in BET, especially seeing RNA vaccines possibly represent NEP.

4 Medical devices

The global medical device industry was valued at \$426 billion in 2018 by Fortune Business Insights and expected to reach \$613 billion by 2025 with a compounded annual growth rate (CAGR)

¹³ <https://www.rapidmicrobiology.com/event/vaccine-safety-pyrogen-and-endotoxin-testing-from-development-through-commercialization>



of 5.4%¹⁴. North America was valued at \$169 billion in 2018. We conducted a workshop (Kerecman Myers et al., 2017) that addressed the opportunities for alternative methods for medical device testing. To date, biological testing of medical devices is mostly done by animal testing, but the International Organization for Standardization (ISO) has committed to at least consider alternative approaches: “In vitro test methods, which are appropriately validated, reasonably and practically available, reliable and reproducible shall be considered for use in preference to in vivo tests.” (ISO 10993-1, 2009).

Pyrogen testing of medical devices is a concern, as many medical devices are inserted into the human body, and pyrogenic properties or contaminations could impact on the patient. Medical device pyrogen testing is an important part of the pyrogen testing market (see below). Noteworthy, pyrogen-contaminated devices also cause an alteration in the hemostatic response when compared to depyrogenized devices (Stang et al., 2014), showing the impact of pyrogen exclusion beyond a febrile reaction. Therefore, pyrogenicity testing should be performed prior to hemocompatibility tests according to ISO 10993-4 in order to exclude hemostatic activation induced by pyrogen contaminations.

The author joined ISO Technical Committee 194 (TC 194) from 1998 to 2003 followed by Albrecht Wendel for about five more years. TC 194 is comprised of working groups that develop and maintain standards and technical reports on the biological and clinical evaluation of medical devices. In parallel, we demonstrated opportunities for pyrogen testing with whole blood MAT (Hasiwa et al., 2007; Mazzotti et al., 2007), especially that it can be carried out directly on the surface of devices without prior elution (for details see Hartung, 2015). A more recent study confirmed this (Stang et al., 2014): “It can be stated that pyrogen detection in eluates completely underestimated the contamination level on the medical devices. However, direct incubation of these contaminated samples in pyrogen-free water with saline concentrate solution in the dynamic rotation model yielded more than 90% pyrogen recovery rates indicated by IL-1b release from whole blood cells”. We succeeded in 1998 in establishing a pyrogen working group in ISO TC 194, which has in the meantime finalized its guidance¹⁵, recommending: “In some cases, the HCPT [Human cell-based pyrogen test] can be a useful alternative to traditional pyrogenicity test methods (rabbit and LAL); however, the rabbit test will need to be retained for detection of pyrogens not detected by the HCPT, including material-mediated pyrogens. Therefore, it is very important that the appropriate method is selected based on the purpose of pyrogen test of medical devices and their materials.”

The guidance lists the following disadvantages of MATs:

- Material-mediated pyrogens that are chemical agents do not operate through the cytokine network to induce a febrile reaction and most likely will not be detected on the HCPT.
- Drugs that interact with monocytes or macrophages (for

example, cytokine receptor antagonists, non-physiological solutions, cytotoxic agents, recombinant proteins with cytokine activity) or the detection system (for example, rheumatic factors), may not be tested with HCPT.

- HCPT may be not applicable to tissue-engineered products containing living cells that release cytokines and chemokines.
- The response to pyrogen in this test can be dependent on the donor of the blood sample or cell conditions. Particularly, whole human blood may vary due to differences in donors age, gender, genetic background (genetic polymorphisms in genes coding for Toll-like receptors cytokine receptors, etc.), safety issues with infected donors, diurnal variation, influence of diet, and other factors which may influence the sensitivity and specificity of the whole blood in vitro tests.
- The whole blood supply system can be a problem.
- HCPT using solid samples directly may be unavailable for routine quality control testing of batches of large finished, sterilized products/devices for presence of pyrogen contamination.
- HCPT using human myelomonocytic cell lines has the disadvantages of time, cost and technical complication for pre-culture and priming of the cells.
- NOTE The only reliable positive control is LPS; controls for assessing interference for other pyrogens is generally unknown.”

Altogether, this is not very encouraging for the use of MAT. In the view of the author, these comments are disproportionate, as several shortcomings could be as well attributed to the rabbit assay or BET, and most do not hold for the majority of medical devices. The key problem of ISO as a consensus process is that individual member’s concerns have to be accommodated as progress can otherwise be blocked.

A major discussion, as evident from the list of disadvantages in the guidance, was about material-mediated pyrogenicity, i.e., whether non-microbial substances as contaminants in medical devices can induce fever reactions. ISO 10993-11, ANNEX G: Material-mediated pyrogenicity lists the following substances, which are known (and verified) to generate a pyrogenic response without being endotoxins:

- endogenous pyrogens (e.g., IL-1, IL-6, TNF α , INF- γ);
- prostaglandin;
- inducers (e.g., polyadenylic, polyuridylic, polybionosinic and polyribocytidylic acids);
- substances disrupting the function of thermoregulatory centers (e.g., LSD, cocaine, morphine);
- uncoupling agents of oxidative phosphorylation (e.g., 4,6-dinitro-o-cresol, dinitrophenol, picric acid);
- N-phenyl- β -naphthylamine and aldo- α -naphthylamine (the febrile mechanism is unknown);
- bacterial exotoxins (e.g., TSST-1, SEA, Spe F, Spe C);
- neurotransmitters (e.g., noradrenaline, serotonin)
- Metals such as nickel salts, in some instances

¹⁴ <https://www.fortunebusinessinsights.com/industry-reports/medical-devices-market-100085>

¹⁵ <https://www.iso.org/standard/71150.html>

This was the reason – not to say instrumentalized – for some members to delay the acceptance of MATs for pyrogen testing despite the fact that neither rabbit tests nor LAL, which are accepted for pyrogen testing of medical devices, have been shown to detect these. Interestingly, at least trinitrophenol was shown in the meantime to be positive in the whole blood-MAT (Banerjee and Mohanan, 2011).

The FDA guidance on the ISO recommendations very much builds on this¹⁶: “*Pyrogenicity: Implants (due to their contact with the lymphatic system), as well as sterile devices having direct or indirect contact with the cardiovascular system, the lymphatic system, or cerebrospinal fluid (CSF) (regardless of duration of contact) and devices labeled as ‘non-pyrogenic,’ should meet pyrogen limit specifications. ... Pyrogenicity information is used to help protect patients from the risk of febrile reaction. There are two sources of pyrogens that should be considered when addressing pyrogenicity. The first, material-mediated pyrogens, are chemicals that can leach from a medical device during device use. ... Pyrogens from bacterial endotoxins can also produce a febrile reaction similar to that mediated by some materials. ... If recommended for consideration per Attachment A, material-mediated pyrogenicity testing is not needed if chemical characterization of the device extract and previous information indicate that all patient-contacting components have been adequately assessed for pyrogenicity. Otherwise, we recommend that you assess material-mediated pyrogenicity using traditional biocompatibility extraction methods (e.g., 50°C for 72 hours; 70°C for 24 hours; or 121°C for 1 hour per ISO 10993-12:2012), using a pyrogenicity test such as the one outlined in the USP 34 <151> Rabbit Pyrogen Test or an equivalent validated method. For devices that contain heat labile or heat sensitive materials (e.g., drugs, biomolecules, tissue-derived components), which may have the potential to undergo deformation or material configuration/structural change at high temperature, extraction at 37°C per ISO 10993-12:2012 is recommended.*”

Borton and Coleman (2018) made an important contribution, showing with a comprehensive review that there is no evidence for such substances relevant to medical devices producing adverse reactions. To say it more clearly, in all my time of working on pyrogens, I have not seen a substance relevant to medical devices inducing pyrogenicity, very similar to the Borton and Coleman assessment, and the strong emphasis of FDA in this respect is difficult to understand.

In 2018, NICEATM and the PETA International Science Consortium Ltd, convened a workshop at the National Institutes of Health (Brown et al., 2021) entitled “*Using the monocyte activation test as a standalone release test for medical devices*”. To the best of my knowledge, this was the first MAT workshop in the US

(followed in 2019 by the U. S. Pharmacopeia (USP) one referred to below). The workshop explored how the FDA’s Medical Device Development Tools (MDDT) program¹⁷ can definitively qualify the use of MAT as a stand-alone pyrogen release test for a specific medical device context of use. Critical to the planning and execution of this workshop was input from key players including FDA Center for Devices and Radiological Health (CDRH), USP, ISO, MAT experts, and medical device companies conducting pyrogenicity testing.

On this occasion, Dr Molly Gosh presented the FDA position¹⁸, i.e., the CDRH requirements to accept the MAT as a replacement for the rabbit pyrogen test and/or BET to meet medical device biocompatibility and sterility testing requirements, and the role of the MDDT Program in meeting these requirements. This included a discussion of how narrowly context of use must be defined for industry consortia planning an MDDT proposal. She presented a number of challenges to the use of MAT (Tab. 3). The personal remarks in the second column in Table 3 might show that the author sees the extent of concerns raised where accepted tests have never undergone similar scrutiny and suffer a number of shortcomings as an enormous roadblock being built. Hopefully, this can be clarified and mitigated in further discussions.

5 Blood transfusions and cellular therapies

Nearly 21 million blood components are transfused annually in the U.S.¹⁹. In contrast to other infused medical products, these are not routinely tested for microbiological contaminations and pyrogens. This includes approximately 4 million platelet units (Brecher and Hay, 2005), though more recent estimates are only at 2 million per year (Alcaina, 2020): “*Unlike red cell or whole-blood components, which are stored at 1 to 6°C, platelets are stored at 20 to 24°C to preserve function and survival. Such storage makes them an excellent growth medium for a broad spectrum of bacteria. Multiple aerobic-culture surveillance studies have demonstrated that 1 in 1,000 to 2,000 platelet units are bacterially contaminated. Thus, 2,000 to 4,000 bacterially contaminated units would be expected to be transfused per year. Estimates of the fraction of cases that result in patient symptoms have been as low as 1 in 10 cases. ... Thus, clinical sepsis would be expected in at least 1 in 10 to 2 in 5 contaminated transfusions (200 to 1,600 cases). National passive-reporting studies from the United States, the United Kingdom, and France ... suggest that one-fifth to one third would result in death (40 to 533 deaths per year)... This translates to a risk of death from a transfusion of a bacterially contaminated platelet unit of between 1 in 7,500 and 1 in 100,000. Clinical observations from university hospitals actively pursu-*

¹⁶ Use of International Standard ISO 10993-1, “Biological evaluation of medical devices – Part 1: Evaluation and testing within a risk management process” Guidance for Industry and Food and Drug Administration Staff Document issued on: June 16, 2016

¹⁷ <https://www.fda.gov/medical-devices/science-and-research-medical-devices/medical-device-development-tools-mddt>

¹⁸ https://www.piscitd.org.uk/wp-content/uploads/2018/09/2_Ghosh_FDA-MDDT_2018.pdf https://www.piscitd.org.uk/wp-content/uploads/2018/09/2_Ghosh_FDA-MDDT_2018.pdf

¹⁹ <https://www.redcrossblood.org/donate-blood/how-to-donate/how-blood-donations-help/blood-needs-blood-supply.html>



Tab. 3: Requirements suggested by the Center for Devices and Radiological Health (CDRH) to accept the MAT as a replacement for the rabbit pyrogen test and/or BET to meet medical device biocompatibility and sterility testing requirements
FDA position presented by Dr Molly Gosh¹⁸ and personal comments of the author.

Considerations for qualification presented by the FDA representative at the workshop	Personal view of the author
<ul style="list-style-type: none"> • Is the proposed test going to replace both bacterial endotoxin and rabbit pyrogen tests? <ul style="list-style-type: none"> – If so, is test qualified for detection of both endotoxin and non-endotoxin pyrogens? – Non-endotoxin pyrogens: <ul style="list-style-type: none"> ◦ Chemical agents (material-mediated pyrogenicity) ◦ Microbial components other than LPS 	<ul style="list-style-type: none"> • Yes <ul style="list-style-type: none"> – Yes (in contrast to BET, which is broadly accepted for medical devices) – There is no evidence for material-mediated pyrogenicity by materials relevant for medical devices; there is a broad body of evidence showing that MAT detects NEP of microbial origin.
<ul style="list-style-type: none"> • How does the endpoint measured in the test relate to the fever response in human which is a complex process? <ul style="list-style-type: none"> – Rabbit pyrogen test detects whole body fever response – Relationship between single/multiple cytokine levels (e.g., IL-1 and/or IL-6) produced in cultures of monocytes vs. fever response in human 	<ul style="list-style-type: none"> • There is clear-cut evidence that monocyte activation upon recognition of pyrogens is translated to IL-1 release (to a lesser extent IL-6 and TNF) resulting in fever. <ul style="list-style-type: none"> – Of rabbits and to Gram-negative LPS – there is considerable doubt for Gram-positive pyrogens. – No strong evidence that the cytokine measured makes a difference.
<ul style="list-style-type: none"> • Is the proposed endpoint the sole outcome measure for assessing the fever response irrespective of the mechanism of action of pyrogens? <ul style="list-style-type: none"> – For, e.g., endotoxin vs. agents that directly affect the thermoregulatory center in the brain vs. uncoupling agents of oxidative phosphorylation 	<ul style="list-style-type: none"> • After 40 years of human MAT there is no indication that another mechanism needs to be covered. <ul style="list-style-type: none"> – While such agents would likely be missed, there is no evidence that they are found in medical devices.
<ul style="list-style-type: none"> • With what types of devices can the proposed test be used? <ul style="list-style-type: none"> – e.g., durable/absorbable devices that include polymers, ceramics, metals, biologics, hydrogels, liquids 	<ul style="list-style-type: none"> • Product-specific validation will be required, but broad applicability has been shown.
<ul style="list-style-type: none"> • Assay Interference Testing <ul style="list-style-type: none"> – Testing to verify that a test article/extract does not interfere with cell system or with the cytokine-specific ELISA 	<ul style="list-style-type: none"> • This would be the same as for other medical products.
<ul style="list-style-type: none"> • Can this test be qualified for use with devices having different regulatory “EU/device” limits? <ul style="list-style-type: none"> – 20 EU/device (for devices in direct or indirect contact with cardiovascular system and lymphatic system) – 2.15 EU/device (for devices in contact with cerebrospinal fluid) – ≤ 0.2 EU/device (for intraocular lenses) 	<ul style="list-style-type: none"> • These pyrogen amounts are not a challenge to MATs. As they have been established for eluates, moving to on-device testing would be even more sensitive. As the assays are quantitative, different thresholds can easily be accommodated.
<ul style="list-style-type: none"> • Are any device-specific method optimizations needed? For example: <ul style="list-style-type: none"> – Use with large versus small surface area devices – Use with device extracts versus direct testing on the device itself – If direct testing on the device: <ul style="list-style-type: none"> ◦ Is the test limited to detecting surface bound pyrogens only? Is this sufficient? ◦ Is there any difference if the test is done under static vs. dynamic incubation conditions? ◦ Can the test detect all pyrogenic extractables/leachables? <ul style="list-style-type: none"> – How comparable is the amount of pyrogenic extractable/leachable that can elute out during the exposure period in this assay vs. in the test extract prepared using ISO 10993-12 extraction condition (e.g. for saline extract prepared by extracting the device in saline at 50°C for 72 hour using an extraction ratio of 3 cm² surface area of the test article/mL of saline) 	<ul style="list-style-type: none"> • Probably as part of the device-specific validation. <ul style="list-style-type: none"> ◦ What does “limited to detecting surface bound pyrogens” mean? This is not a limitation but a new opportunity. What else should it detect? ◦ Probably, but this would be part of the product-specific validation. ◦ All known ones, but this will be always an open question. <ul style="list-style-type: none"> – This will depend on the medical device. So far, the direct incubation seems to be more sensitive in the cases studied so far, because elution is partial, leads to dilution of pyrogens, and there are presentation effects for NEP on surfaces that increase their potency.

Considerations for qualification presented by the FDA representative at the workshop	Personal view of the author
– Optimization of treatment period to increase test sensitivity	– So far, no need to modify incubation periods for direct incubation.
• Are there any chemicals or device designs incompatible with the test system?	• While no evaluated substances so far impeded pyrogenicity testing, this will have to be evaluated in product-specific validation.
• How can positive controls be selected to confirm that the proposed test can distinguish between positive and negative responses for non-endotoxin pyrogens?	• We still lack NEP reference materials, but various kits contain NEP positive controls. In the view of the author, lipoteichoic acids from Gram-positive bacteria are most promising, but an interactive process with a biological standard producer will be needed.
<ul style="list-style-type: none"> • What qualification data already exist for the proposed test, and what data gaps still need to be filled? <p>– Chemical domain space relevant to medical device materials as well as the domain space for combination products (device-drug and device-biologic)</p> <p>– Comparative data: MAT/RPT and LAL tests/human outcomes</p>	<ul style="list-style-type: none"> • See the various articles cited here and in Hasiwa et al. (2013) and Hartung (2015); it appears that largely product-specific validation is needed. <p>– A database for MAT-compatible and non-compatible materials would be desirable. So far, compatibility with a number of solid materials and cell preparations has been shown.</p> <p>– Such data are scarce for rabbit and Limulus; there are examples where the patient reaction was not anticipated by the traditional testing, but typically companies are not willing to share these batches. Stability of NEP in products is not clear and storage/transport for testing is complicated.</p>

BET, bacterial endotoxin test, see LAL; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; IL, interleukin; LAL, Limulus amoebocyte lysate assay, see BET; LPS, lipopolysaccharide, endotoxin; MAT, monocyte activation test; NEP, non-endotoxin pyrogen; RPT, rabbit pyrogen test; TNF, tumor necrosis factor

ing suspected cases of platelet-related sepsis confirm these estimates. Ness et al. from Johns Hopkins University, reported a fatality rate of 1 in 17,000 with pooled whole-blood-derived platelets and 1:61,000 with single-donor apheresis-derived platelets Similarly, University Hospitals of Cleveland observed a fatality rate of 1 in 48,000 per random platelet unit The French BACTHEM study documented a fatality rate due to bacterially contaminated platelets of 7 per 10⁶ (1 in 140,000)²⁰. According to CDC²⁰, approximately 1 in 1,000-3,000 platelet units may be contaminated with bacteria with transfusion-transmitted sepsis recognized and culture-confirmed in at least 1 of 100,000 recipients (with immediate fatal outcome in 1 in 500,000 recipients). The actual risk of transfusion-associated sepsis is likely higher, as infections due to contaminated blood products are underreported. Several studies showed that bacterial contamination of donated blood was 0.2%, 0.15%, and 0.1% in the US, UK, and France, respectively (Brecher and Hay, 2005). Less developed countries face higher rates of contamination, e.g., bacterial contamination was observed in 9% of the blood and blood components in Ethiopia (Agzie et al. 2019).

As summarized earlier (Hartung, 2015), the whole blood MAT sensitively detects such contaminations as well as bacterial pyrogens in these products. While there is some progress towards sterility control, the opportunity of the MAT has not been leveraged yet. We suggested MAT validation for this purpose to FDA in 2018 but were not selected for funding.

Microbial safety of advanced cellular therapeutics for transplantation and regenerative medicine is a similar concern, as contamination can occur during collection or after *in vitro* manipulation with reported rates in various types of progenitor cell products ranging from 0.2 to more than 25%, averaging about 3% (Stormer et al., 2019).

6 Air-borne pyrogens

Except for a small study related to particulate matter and asthma (Negherbon et al., 2017), I am not aware of new developments in the area of assessing air-borne pyrogens using MATs since 2015.

7 Nanomaterials

The concern about pyrogen contamination of nanomaterials (Dobrovolskaia et al., 2010; Hartung, 2010a; Hartung and Sabbioni, 2011) is increasing, and there is increasing awareness that rabbit tests and especially BET are not adequate (Li and Boraschi, 2016; Neun and Dobrovolskaia, 2017; Li et al., 2017a; Jin et al., 2018). Li et al. (2017b) showed that LPS bound to gold nanoparticles activates monocytes. Otherwise, the author is not aware of relevant publications since 2015 referring to MAT pyrogen testing of nanomaterials. Another missed opportunity.

²⁰ <https://www.cdc.gov/bloodsafety/bbp/bacterial-contamination-of-platelets.html>



8 Regulatory acceptance of MATs

European Pharmacopoeia (EurPhar) was the first to accept MATs in 2010. In 2016, EurPhar revised the general chapter *Monocyte-activation test 2.6.30*²¹ to make it more widely useable by stakeholders and thus facilitate a reduction in testing on laboratory animals after a wide-ranging consultation with industry representatives, academics, regulatory authorities and Official Medicines Control Laboratories: “*The MAT offers significant advantages over animal testing: based on the human fever response, it provides a more relevant prediction of pyrogenic activity than the RPT, it can detect endotoxin and non-endotoxin pyrogens and is applicable to a greater variety of products than the RPT; moreover, it is more accurate as well as more cost- and time- effective than the RPT.*” Chapter 2.6.8 on pyrogens now encourages the replacement of RPT by MAT: “*Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test (2.6.30).*”

Noteworthy, a new *Test for bacterial endotoxins using recombinant factor C 2.6.32* was also created as a standalone chapter, not referenced in any monograph. It describes a BET that uses a rFC based on the gene sequence of the horseshoe crab, and a fluorimetric endpoint detection method. For now, only the fluorimetric method is described, as the rFC kits currently available on the European market and most of the available scientific data are based on this method.

In keeping with EU legislation (Hartung, 2010b) and its national implementation, the Paul-Ehrlich-Institute in Germany announced in March 2019 that the German state authorities would no longer accept the use of the RPT. At the time, only one larger contract research organization using about 6,000 rabbits per year and some smaller places with together 400 rabbits were active. Other EU member states are expected to follow, thereby eliminating pyrogen testing on animals in Europe (see below). Producers are changing to BET or MAT or moving testing into non-European countries.

FDA’s acceptance of the MAT first took place in 2012²², stating “*Product-specific validation is necessary to establish whether a particular test substance or material is appropriate for evaluation of the monocyte activation method. The validation should include, but is not limited to, interference testing, accurate detection of pyrogen in individual test samples, and, for devices, ability of test system to provide direct contact to the monocytes.*” This was then expanded in 2016 within their Guidance for Industry and Food and Drug Administration Staff: Use of International Standard ISO 10993-1, *Biological evaluation of medical devices – Part 1: Evaluation and testing within a risk management process*²³. The U.S. Pharmacopoeia General Chapter <151> (“Pyrogens”) allows the use of a “*validated, equivalent in vitro pyrogen or bacterial endotoxin test*” in place of the RPT (USP, 2017) “*where appropriate*”. Following this, the United States Pharmacopoeial Convention

in 2019 held a symposium “*Future of endotoxin and pyrogen testing; Reference standards and procedures*”, where the author had the privilege to talk, with three sessions:

1. Endotoxin standards: current test reference standards, intended use, need for new standards
2. Alternative methods for endotoxin testing
3. Pyrogen tests – rabbit and MAT. Non-animal alternatives and standards

The Russian pharmacopoeia and Indian pharmacopoeia added MAT in 2018. The MAT is now also an official method in Brazil: It was recognized by CONCEA (National Council of Animal Experimentation Control), as reported by BraCVAM. The next step will be to include it in the Brazilian pharmacopoeia. The MAT has apparently been included in the Chinese Pharmacopoeia Vol 1 General Principles 1142 in Revision 2015 (not yet translated), and the method is under evaluation in Japan. So, a lot has happened with regard to regulatory acceptance since 2015. Thus, we are reaching an almost global acceptance of the MAT, a prerequisite for its broad use in a globally acting industry.

9 The pyrogen testing market

It is the strong belief of the author that economic aspects are not sufficiently discussed in relation to alternative methods. Therefore, we have twice addressed this in this series of articles (Bottini and Hartung, 2009; Meigs et al., 2018) as well as in one article on globalization (Bottini et al., 2007). In 1995, pyrogen testing did not seem to be a frontline issue for safety science or alternatives to animal testing. In particular, the BET supply industry had just formed, with three companies dominating the market (outside Japan) at an estimated \$150 million annual turnover (James Cooper, personal communication, cited in Hartung, 2015). Replacement of the rabbit pyrogen test with the BET began in 1983 with its introduction into the USP 29 monographs for radiopharmaceuticals and for five USP pharmaceutical waters²⁴. The discussion about shortcomings of the BET simply ebbed away, and the need for something new was not as apparent to many, and is in some cases not until today, certainly fostered by the shiny brochures and training provided by the LAL industry.

A bit surprising though was that rabbit numbers did not seem to decrease as much as the rising BET industry numbers suggested. The costs for the BET were considerably below one tenth of the animal tests – but though I am not aware of statistics, the rabbit testing market was never ten times the Limulus market. The reason is simply that while the BET did its share to bring some animal testing down, it was first of all an enabling technology that was used where the rabbit test could not be used, e.g., short-lived radiopharmaceuticals and testing of water. Foster T. Jordan, the CEO of Endosafe, once told me that 90% of BET were done on

²¹ https://www.edqm.eu/sites/default/files/press_release_pheur_comm_155th_session_mat.pdf

²² <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-pyrogen-and-endotoxins-testing-questions-and-answers>

²³ <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/use-international-standard-iso-10993-1-biological-evaluation-medical-devices-part-1-evaluation-and>

²⁴ Nice freely available summary of BET by James F. Cooper and Joseph C. Hung: https://pharmacyce.unm.edu/nuclear_program/freelessonfiles/vol14lesson5.pdf

Tab. 4: The global pyrogen testing market as assessed by Marketsandmarkets

Rates in black are valuations and in blue growth predictions.

Report	CAGR	2014	2016	2019	2020	2021	2025
2015	12.2%	462 m\$		823 m\$			
2016	12.2%		610 m\$			1086 m\$	
2020	12.7%				927 m\$		1689 m\$

CAGR, compounded annual growth rate, i.e., the rate of return that would be required for an investment to grow from its beginning balance to its ending balance, assuming the profits were reinvested at the end of each year of the investment's lifespan.

water samples – certainly not an application where rabbit testing was the large-scale competition. So, the BET found its market without taking too much away from the rabbit testing market. This explains why the European statistics from 2008 and 2011 still showed 160-170,000 rabbits being used for pyrogen testing in Europe alone.

Based on the MarketsandMarkets™ report “*Pyrogen Testing Market by Product & Service (Assays, Kits, Reagents, Instruments, Services), Test Type (LAL, Chromogenic, Turbidimetric, Gel Clot, In Vitro, Rabbit), End User (Pharmaceutical, Biotechnology, Medical Device), Region – Global Forecast to 2025*”²⁵, the market size is projected to reach \$1,689 million by 2025 from \$927 million in 2020, at a 12.7% CAGR. As vaccine manufacturing is expected to increase due to COVID-19, BET will be an important quality assurance measure to ensure patient safety here too. Table 4 shows the change in estimates by Marketsandmarkets over time; while the author cannot judge the quality of these data (though my personal impression is that numbers are too high), assuming a consistency in approach, this illustrates an enormous growth of the field over time.

Their 2016 report states it is based on annual reports, SEC filings, investor presentations, American Society of Microbiology, Association of Pharmaceutical Research and Development, Biotechnology and Biological Science Research Council, Canadian Institute of Health Research, Department of Biotechnology, European Federation of Biotechnology, Parenteral Drug Association, World Bank, World Intellectual Property Organization, expert interviews, and MarketsandMarkets analysis. It reports market shares of 59% for LAL tests, 30% for *in vitro* pyrogen tests and 11% for rabbit tests with 43% for kits and reagents, 37% for services, and 20% for instruments. The majority of testing with 76% is for pharmaceuticals and biologicals, 15% for medical devices, with a geographical split of 38% North America, 31% Europe, 20% Asia, and 10% the rest of the world.

10 Animal use for pyrogen testing

As mentioned already, pyrogenicity testing in Europe consumed 160,000 to 170,000 rabbits in 2008 and 2011, respectively. One year ago, the European Commission published animal use statistics for 2015-2017 (Busquet et al., 2020): Pyrogenicity tests decreased to 46,553 in 2015 and to 35,172 in 2017. In total, 14 countries reported animal-based pyrogenicity testing, with highest numbers in Austria and Spain (54.8%, 2015-2017) followed by Germany, France and Italy (together > 90% of quality control pyrogenicity testing). In many cases, the numbers dropped over the three years. Noteworthy, the UK just published that no rabbit pyrogen tests were reported at all in 2019, compared to 638 tests in 2018. Similar developments in Germany were described above. This is all reason to celebrate, but the questions remain: Why did it take so long? And why is there still rabbit testing ongoing?

Ultimately, a number of organizations started to increase pressure on the industry and on regulators to make the transition. For example, the EPAA (the European Partnership on Alternative Approaches to Animal Testing) made pyrogenicity testing their new focus in 2018²⁶ and several animal welfare organizations have campaigned for this, e.g., in Europe^{27,28,29} and in the US^{30,31,32}. This has closed the gap between acceptance and implementation of alternative methods.

11 Conclusions

The ongoing replacement of an enormous number of rabbit tests with a human cell-based assay, which actually outperforms the animal test, drawing closer to completion is one of the biggest successes of alternative methods and their validation. It can serve as a poster child for the field, as the discovery of the pathophysi-

²⁵ <https://www.marketsandmarkets.com/Market-Reports/pyrogen-testing-market-255266155.html>

²⁶ <https://ec.europa.eu/docsroom/documents/33127/attachments/1/translations/en/renditions/native>

²⁷ <https://www.aerzte-gegen-tierversuche.de/de/helfen/kampagnen/3204>

²⁸ <https://worldanimalsvoice.com/2020/07/09/we-want-to-stop-the-pyrogen-test-for-rabbits/comment-page-1/>

²⁹ <https://vgt.at/presse/news/2018/news20180423mn.php>

³⁰ <https://www.peta.org/blog/fda-non-animal-pyrogen-tests/>

³¹ <https://www.piscltd.org.uk/our-work/pyrogenicity/>

³² <https://www.peta.org/issues/animals-used-for-experimentation/pyrogenicity/>



ology of pyrogenicity allowed tests based on the human fever reaction to be built.

The developments benefitted from patenting at an early stage when this was a prerequisite for engaging companies to invest into this opportunity. However, it turned out that these expectations were not met owing to the lengthy process of validation and acceptance and, at some point, patenting became a drawback of the whole blood MAT. Other variants of MATs, though with some disadvantages and less supporting data, were favored to avoid the patents, and even the whole blood MAT licensor invested into other MATs. In regulatory testing, there is no market before regulatory acceptance. If we want technology providers to be a driving force of alternative methods, we have to rethink this business model.

In order for MATs to take their rightful role in safety assessment, we need an open discussion about NEPs and other shortcomings of the BET. A centralized, evidence-based repository of such information would move the discussion from anecdotal and case-by-case problem-solving to a gap analysis and targeted development of new strategies. If we see the figures on the pyrogen market, it is clear that such proposals will find major resistance, but if we expand the calculations of numbers of people harmed and possibly bring liability to bear, this might change. Nobody can claim innocence about the safety gaps of the BET with the mounting evidence and the availability of alternatives that overcome them. It is the right strategy for the BET industry to embrace and not antagonize the new methodologies as we see in part happening.

For each area of application, it takes champions to promote the new assay, as we found especially with our colleagues at the German Paul-Ehrlich-Institute. These champions are missing in some areas with obvious opportunities to improve patient safety such as blood transfusion and cell therapy, nanomaterials, and to assess exposure such as air-borne pyrogen testing.

Altogether, however, I underestimated the resistance of the safety testing field to change. The promise of the European legislation forcing the use of an alternative, if one is practically available, remains an empty shell without the tools to enforce this. It should not be permitted to carry out an animal test once an alternative has been accepted. Industry trying to avoid change often argues a continuing need for the animal test in other countries. Okay, then do the test where it is required, and do the alternative where this is the standard! This would provide us in turn with many direct comparisons between the old and the new tests. We should also not accept animal tests done abroad for registrations in countries where the test has been replaced. Such measures would make the companies engines of change trying to avoid double testing.

The whole blood MAT has shaped my career and been a continuous learning opportunity for 25 years. In many areas, it confronted me with real-life applications outside my ivory tower of research. I would not have dreamt of getting involved with safety and batch release testing, kit development, the many workplaces including air and space industry for airborne pyrogens, childhood asthma, nanoparticle toxicology, blood transfusion and cellular therapies, etc. without it. It gave my research new directions, such as more than 100 publications on lipoteichoic acids, which I consider the Gram-positive pyrogen until proof of the contrary; it brought me to validation and to ECVAM and

a broader role in alternatives to animal testing. It also taught me patience – not my strength – but it is difficult to see missed and delayed opportunities over 25 years and still continue to push forward. Major reductions in rabbit use in Europe are a first step, but the best for the MATs is yet to come...

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Conflict of interest

Thomas Hartung held patents as inventor of the whole blood pyrogen test and the use of cryopreserved blood, which were licensed to Merck-Millipore; he continues to consult them and receives royalties from Merck-Millipore from sales of the kit version.

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