MONOCYTE ACTIVATION TEST AS REPLACEMENT OF ANIMAL-BASED METHODS FOR THE *IN VITRO* ASSESSMENT OF HUMAN VACCINE PYROGENICITY

Marilena Paola Etna (a), Fabiana Rizzo (a), Daniela Ricci (a), Ramona Ilari (b), Martina Severa (a), Valentina Salvati (c), Andrea Gaggioli (c), Ingo Spreitzer (d), Christina von Hunolstein (c), Eliana Marina Coccia (a)
(a) Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy
(b) Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy
(c) National Centre for Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome, Italy
(d) Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Langen, Germany

Introduction

The quality of vaccines for human use is assured by several periodic controls performed by the manufacturer under Good Manufacturing Practices on the raw materials and final vaccine formulation. Moreover, in accordance with the European Directive 2001/83/EC on medicinal products for human use, marketing authorization also requires the testing of critical parameters on the final vaccine formulation by the European Official Medicine Control Laboratory (EU OMCL). In this context, safety and potency are key parameters that needs to be monitored by careful and reliable testing that often involve a very significant number of animals.

Being pyrogens one of the principal causes of fever and febrile reactions in humans, it is of key importance to control pyrogenicity in parenterals, including vaccines. Moreover, pyrogens can induce also other physiological reactions ranging from septic shock to multi-organ failure and, sometimes, even death (Prajitha *et al.*, 2018).

The most pyrogenic, abundant and stable exogenous pyrogen is the endotoxin (lipopolysaccharide, LPS) from Gram-negative bacteria (endotoxin, i.e., the "toxin" is part of the bacterium, and not actively secreted). Nevertheless, virtually all gram-positive/gram-negative bacteria, either alive or their break-down products like LPS, muramyl peptide derivatives and peptidoglycans – all of them Pathogen Associated Molecular Patterns (PAMPs), sometimes called non-endotoxin pyrogens (NEP) –, behave as (weaker) endotoxins. In addition, other microbial-derivates, as fungal products (mannan and glucan components of *Candida albicans*), viral RNA, enterotoxins (*Staphylococcus aureus*) and erythrogenic toxins (Group A streptococcus) may act as pyrogens.

Currently, different tests are foreseen in European Pharmacopoeia (Ph. Eu.) for pyrogen testing namely the Rabbit Pyrogen Test (RPT, Chapter 2.6.8), the Bacterial Endotoxin Test (BET, Chapter 2.6.14), the Recombinant Factor C test (rFC, Chapter 2.6.32) and the Monocyte Activation Test (MAT, Chapter 2.6.30).

The RPT is considered the gold standard for pyrogen testing. It is an *in vivo* method where variations of body temperature are measured in rabbit after the injection of the solution to be examined. The rabbit model has been chosen because of the similarity with the human response to endotoxin and this test allows the qualitative measurement of both endotoxin and NEP (Greisman & Hornick, 1969). Another widely used assay is the BET, also known as Limulus Amoebocyte Lysate (LAL) assay. This limit/qualitative test is able to detect only endotoxin contaminants, by using the amebocyte lysate from the horseshoe crab Limulus polyphemus. Different methods are exploitable for this test: the gel-clot method; the turbidimetric method and

the chromogenic method. Although the existence of alternative methods, RPT and BET – both animal-based tests – are still widely diffused in routine testing of medicinal products and, thus, they poorly adhere to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Indeed, the intention of this directive was to improve and enforce animal welfare by giving stricter advice on how to transfer these measures into national law.

The theoretical basis of the above-mentioned EU Directive has been firstly postulated in 1958 in the book "The Principle of Human Experimental Technique" authored by Rex Burch and William Russell where the three principles of replacement, reduction and refinement –known as the 3Rs principle – were introduced (Russell & Burch, 1959). Accordingly, in the field of vaccines, technical progress in analytical methods and their application are currently implemented in quality strategy to adhere to 3Rs principle (Akkermans *et al.*, 2020).

Besides ethical reasons, *in vitro* assays represent a more suitable alternative than animal-based methods by improving the evaluation of critical attributes for the product quality in terms of low variability, high sensitivity, and reduction of time and costs.

In line with the 3Rs principle and thanks also to the advance in science and technology, some alternatives for pyrogenicity testing have been developed. In particular, the rFC test (evolution of LAL test using a recombinant version of the protein purified from the horseshoe crab blood) recently introduced in the Ph. Eu. allows the quantitative measurement of endotoxin contents, thus representing a non-animal alternative for BET. Another attractive possibility for the replacement of the RPT is the MAT, a semi-quantitative/quantitative test, based on the capacity of human monocytes or monocytic cells to release endogenous mediators of inflammation, after the stimulation with either endotoxins or NEP (Hartung, 2021).

This test was originally described and inserted in Ph.Eu. as an alternative for testing parenterals however, in the last years it was applied for vaccine testing too (Figure 1).

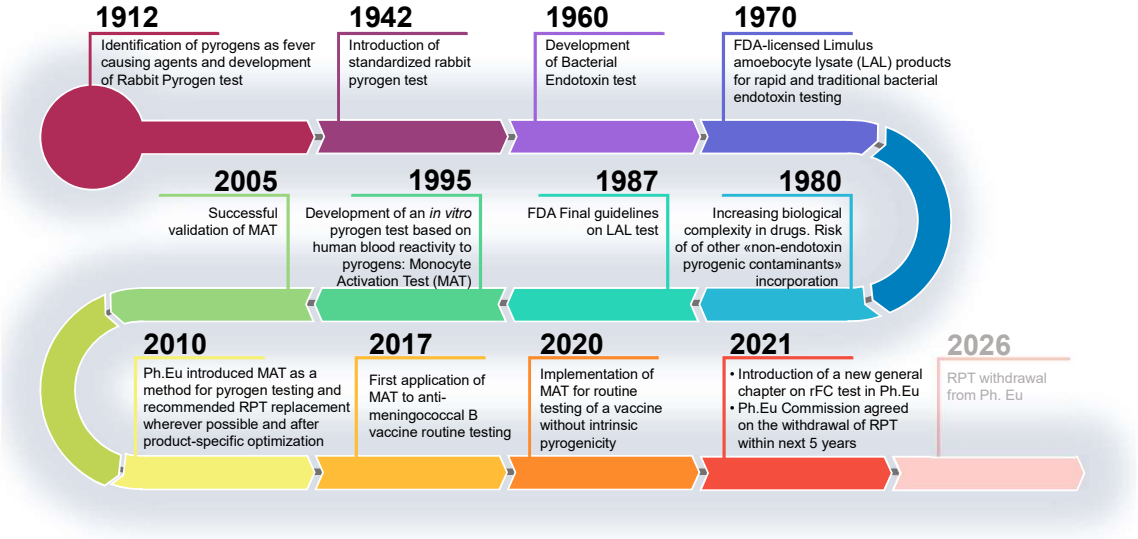

Figure 1. Main achievements in pyrogen testing

First application of MAT to an inherently pyrogenic product: the case of an anti-meningococcal B vaccine

Although rabbits and man similarly react to endotoxins, the response to high levels of pyrogens became more pronounced in human with respect to rabbits (Greisman & Hornick, 1969), likely dependent of differences in the two immune systems.

In light of these differences, the presence of several PAMPs in vaccine formulations has recently raised concerns about the reliable applicability of RPT for pyrogen detection. Accordingly, this issue came up when RPT was applied in the routine testing of an anti-meningococcal B vaccine (anti-MenB), an aluminum hydroxide-adsorbed multicomponent subunit vaccine containing three *Neisseria meningitidis* recombinant proteins, namely the Neisserial adhesin A (NadA), the Heparin-Binding Antigen (NHBA) and the factor H binding protein (fHbp) and the *N. meningitidis* Outer Membrane Vesicles (OMV) (Valentini *et al.*, 2019). The presence of OMV from serogroup B *N. meningitidis*, which contains meningococcal LPS (endotoxin) and several lipoproteins, porins, peptidoglycan and muramyl peptides (NEP) confers to the product inherently pyrogenic properties that made it difficult to adapt the RPT, which resulted in high variability and several false positive results (Vipond *et al.*, 2016). Similarly, also LAL test was not suitable for the anti-MenB vaccine given the presence of NEP (Valentini *et al.*, 2019). Challenges earned with RPT and LAL assays together with growing interest and regulatory requirements in substitution of animal-based methods with *in vitro* approaches opened to the possibility to optimize the MAT as an alternative test for the pyrogen testing of MenB vaccine. Accordingly, thanks to the collaboration of the manufacturer with the OMCLs, MAT was successfully adapted, optimized and validated for assessing pyrogenicity of MenB vaccine batches in routine testing (Valentini *et al.*, 2019). Interestingly, building on anti-MenB vaccine experience, recently the MAT was developed and proposed as replacement of RPT for the consistency/safety testing of Shighella spp. vaccines based on Generalized Modules For Membrane Antigens (GMMA) comprising OMV from genetically modified Gram-negative bacteria and thus being inherently pyrogenic (Carson *et al.*, 2021) (Figure 2).

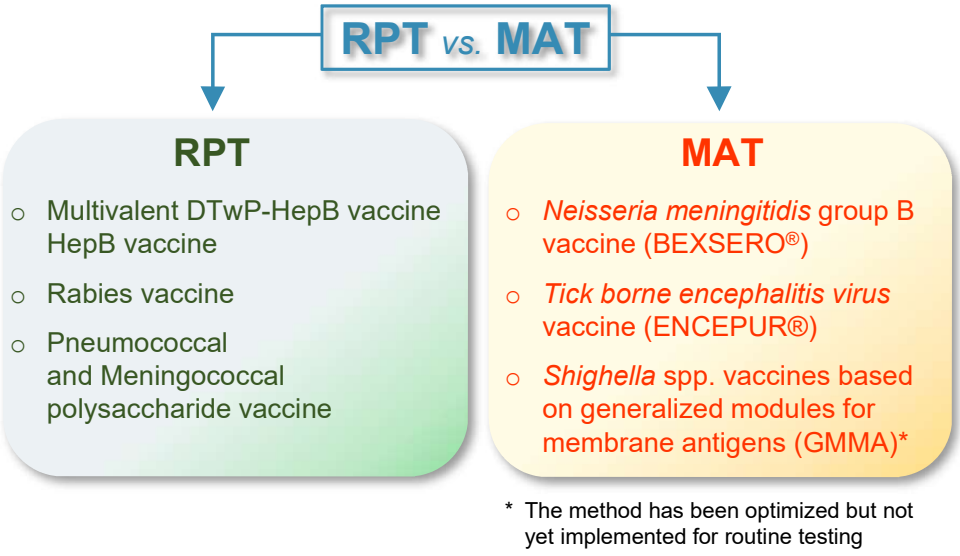

Figure 2. Application of RPT and MAT in the batch release of the human vaccines

For intrinsic pyrogenic products the MAT reference lot comparison method was identified as the most suitable. In particular, the estimation of the relative pyrogenicity (RR) of MenB vaccine batches is carried out on four different peripheral blood mononuclear cells (PBMC) donors through the comparison against a vaccine batch used as reference standard. A regression analysis on a multi-dilution dose-response is performed, starting with the estimation of individual slopes and intercepts (for the various preparation) in order to build an ANOVA (Analysis of Variance) table and to estimate the RR. The final result is the Geometric Mean of the four RRs obtained for the different donors are included in the analytical session.

In 2017, MAT assay optimized for the MenB vaccine was approved by the European Medicines Agency (*see* Figure 1) and recently implemented for batch release and post-marketing surveillance controls at ISS by the MAT Unit of Department of Infectious Diseases in collaboration with the National Centre for Control and Evaluation of Medicines.

Optimization, validation, and implementation of MAT for a not intrinsically pyrogenic vaccine: proposed modifications to MAT monograph

In recent years, a wide-ranging collaborative project funded by Innovative Medicine Initiative 2 (IMI 2), namely "Vaccine batch to vaccine batch comparison for consistency testing" (VAC2VAC), aims to provide the proof of concept of consistency approach for batch release of vaccine by means of *in vitro* analytical methods (http://www.vac2vac.eu/). The ambition of this public-private consortium is to develop and optimize non-animal-based model for demonstrating vaccine batch consistency, safety and efficacy. Accordingly, one of the objectives of ISS Unit within VAC2VAC project is to optimize the MAT for the Tick-Borne Encephalitis Virus (TBEV) vaccine as alternative for the replacement of the currently used RPT test.

Indeed, the anti-TBEV vaccine is among products for which both WHO technical report series and Ph. Eu. product specific monograph foresees the assessment of the pyrogen content as safety test prior to batch release. The anti-TBEV vaccine is composed by the TBEV inactivated by formaldehyde, as active substance, adsorbed onto aluminium hydroxide (Kubinski *et al.*, 2020). Although the active substance by itself does not contain pyrogenic molecules – thus resulting a non-intrinsically pyrogenic product – the production process entails some critical steps, namely the embryo harvest from chicken eggs or the virus propagation that could expose to the risk of bacterial, viral or cellular contaminants entering the final product.

Historically, the pyrogen content of the anti-TBEV vaccine was monitored and assessed by RPT. The possibility to replace the RPT with the MAT when testing for pyrogenicity the TBEV vaccine, was investigated at ISS. Both quantitative Method A (pyrogen content expressed as exact amount of equivalent of endotoxin unit present in the product) and semi-quantitative Method B (pyrogenic level of the product expressed as above or below an established threshold) were used. In this particular case, dealing with a vaccine without intrinsic pyrogenicity and for which the requisite is "not pyrogenic", it was clear that an adaptation of the methods A and B validity criteria was necessary to fulfil at the best Ph.Eur. chapter 2.6.30 requirements (Etna *et al.*, 2020). Along the product-specific optimization it was demonstrated that the pyrogen level of the anti-TBEV vaccine can be established by the MAT with a satisfactory precision as evaluated by repeatability and intermediate precision of the method. However, the experience of MAT adaptation to a not intrinsically pyrogenic vaccine opens the door to: i) overcome the restriction of curve linearity with regards to the product dilution range and, ii) foresee the use of Assay Sensitivity instead of Limit of Detection for the definition of the contaminant limit concentration and product maximum

valid dilution to be tested through MAT (Etna *et al.*, 2020). Of note, the MAT for the anti-TBEV vaccine, optimized and validated at ISS, has been successfully implemented for routine testing by the manufacturer. Moreover, during the MAT optimization, few issues related to Method A and B application came up and were discussed with pharmacopoeia experts leading to an inquiry of revision of MAT chapter 2.6.30 forwarded to the Ph. Eu. Commission (*see* Figure 1).

In line with this, a request of the Italian delegation was submitted at 134th meeting of Group 15 of Ph. Eu to replace in the Ph.Eur. monograph 1375 "tick-born encephalitis vaccine (inactivated)" the current RPT with the MAT (Figures 1-2).

Past and future of pyrogenicity test

In the last century pyrogen research and testing was mainly focused on endotoxin, as LPS. Therefore, RPT was without a reliable competitor up to 25 years ago when Hartung and Wendel developed a human whole blood cytokine release assay to detect human-relevant pyrogens or PAMPs, as they are called nowadays (Hartung & Wendel, 1995). A long time was, however, necessary from the development to the implementation of MAT into the Ph. Eu. (MAT Monograph 2.6.30, EDQM, 2010) (*see* Figure 1). Since then, MAT was employed as a substitute for detecting Gram-negative endotoxins and NEP alike in injectables on a case-by-case basis. Nevertheless, in spite of positive feature and strength of MAT assay and its applicability to a wide portfolio of parenteral for human clinical use, the 90% of pyrogen testing is still covered by LAL and RPT. However, in the case of vaccine, the applicability of LAL is limited by three main factors: i) the interference of aluminium hydroxide, a widely used adjuvant boosting the immune response; ii) the content of many pyrogenic components different from LPS; and iii) the broad presence of glucans, which are common in fungi but also in cellulose composing filters, that may activate LAL cascade reactions leading to false-positive signals. To our opinion the number of these limitations it is likely to increase given to the ongoing and never-ending development of vaccine and vaccine formulation. At present, human vaccines, whose batch release foresees the pyrogen testing by RPT and MAT, are listed in Figure 2. However, different other types of vaccines are developed and include the following categories: recombinant microbes, purified antigen or so-called subunit vaccines, synthetic antigen vaccines, RNA and DNA vaccines. This new generation of vaccines often requires a delivery carrier (nanoparticles, viral vectors, etc.) and adjuvants displaying strong immunomodulatory capacity, enhanced and long-lasting protective feature (Pilkington *et al.*, 2021). As a consequence, the pyrogenicity test will require continuous update and implementation to face not only with the potential "canonical" pyrogenic contaminants that can be introduced accidentally during the manufacturing process but also with the intrinsic nature of the vaccine itself. For instance, the recent introduction of the so-called nucleic acid nanoparticles (NANPs)-based vaccine as well as the use of adjuvants or amplifiers – such as metabolic and epigenetic modulators (Dominguez-Andres *et al.*, 2020) – enlarges the portfolio of vaccine components that can trigger the innate immune response. Nevertheless, in this context, the versality and broad capacity of human PBMC to detect and sense a wide variety of PAMPs supports the setting for a "next generation" MAT able to evaluate also excessive unwanted pro-inflammatory features of NANPs, novel adjuvant or amplifier formulations (Dobrovolskaia & Afonin, 2020).

Take home message

MAT is an ever-green assay, whose potential applications are not yet fully exploited for testing both extrinsic and intrinsic pyrogenicity of classical and novel vaccine formulation. In addition of avoiding the use of animal models, the immunological power of this assay relies on the possibility to investigate *in vitro* the main immune cells present in whole blood or PBMC. This represents a win-win feature that makes MAT a malleable *in vitro* experimental setting ready to sense all PAMPs and to face with the forthcoming vaccine formulation to predict their inflammatory nature as well as possible bias of the induced immune response.

Acknowledgments

The study related to the application of MAT to assess the pyrogenicity of TBEV was performed in context of the VAC2VAC consortium, which received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 115924. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA. We thank the VAC2VAC consortium also to create a stimulating and supportive environment that allowed us to collaborate with colleagues from Accademia, Company and Regulatory Agency involved in the development and implementation of alternative methods for lot release for human and animal vaccines. A special thanks to Dr. Elena Giacomini, a retired colleague of us, who strongly contributed to optimize MAT.

References

Akkermans A, Chapsal JM, Coccia EM, Depraetere H, Dierick JF, Duangkhae P, Goel S, Halder M, Hendriksen C, Levis R, Pinyosukhee K, Pullirsch D, Sanyal G, Shi L, Sitrin R, Smith D, Stickings P, Terao E, Uhlrich S, Viviani L, Webster J. Animal testing for vaccines. Implementing replacement, reduction and refinement: challenges and priorities. *Biologicals* 2020;68:92-107. doi:10.1016/j.biologicals.2020.07.010

Carson D, Myhill S, Palmieri E, Necchi F, Rijpkema S, Micoli F, Nordgren IK, Rossi O, Vipond C. Development of a Monocyte Activation Test as an alternative to the Rabbit Pyrogen Test for mono- and multi-component Shigella GMMA-based vaccines. *Microorganisms* 2021;9(7):1375. doi:10.3390/microorganisms9071375

Dobrovolskaia MA, Afonin KA. Use of human peripheral blood mononuclear cells to define immunological properties of nucleic acid nanoparticles. *Nat Protoc* 2020;15(11):3678-98. doi:10.1038/s41596-020-0393-6

Dominguez-Andres J, van Crevel R, Divangahi M, Netea MG. Designing the next generation of vaccines: relevance for future pandemics. *mBio* 2020;11(6):e02616-20. doi:10.1128/mBio.02616-20

Etna MP, Giacomini E, Rizzo F, Severa M, Ricci D, Shaid S, Lambrigts D, Valentini S, Galli Stampino L, Alleri L, Gaggioli A, von Hunolstein C, Spreitzer I, Coccia EM. Optimization of the monocyte activation test for evaluating pyrogenicity of tick-borne encephalitis virus vaccine. *ALTEX ALTEX - Alternatives to animal experimentation* 2020;37(4):532-44. doi:10.14573/altex.2002252

Greisman SE, Hornick RB. Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. *Proc Soc Exp Biol Med* 1969;131(4):1154-1158. doi:10.3181/00379727-131-34059

Hartung T. Pyrogen testing revisited on occasion of the 25th anniversary of the whole blood monocyte activation test. *ALTEX - Alternatives to animal experimentation* 2021;38(1):3-19. doi:10.14573/altex.2101051

Hartung T, Wendel A. Detection of Pyrogens using human whole blood. *ALTEX - Alternatives to animal experimentation* 1995;12(2):70-75.

Kubinski M, Beicht J, Gerlach T, Volz A, Sutter, G, Rimmelzwaan GF. Tick-borne encephalitis virus: a quest for better vaccines against a virus on the rise. *Vaccines* (Basel) 2020;8(3):451. doi:10.3390/vaccines8030451

Pilkington EH, Suys EJA, Trevaskis NL, Wheatley AK, Zukancic D, Algarni A, Al-Wassiti H, Davis TP, Pouton CW, Kent SJ, Truong NP. From influenza to COVID-19: Lipid nanoparticle mRNA vaccines at the frontiers of infectious diseases. *Acta Biomater* 2021;131:16-40. doi:10.1016/j.actbio.2021.06.023

Prajitha N, Athira SS, Mohanan PV. Pyrogens, a polypeptide produces fever by metabolic changes in hypothalamus: Mechanisms and detections. *Immunol Lett* 2018;204:38-46. doi:10.1016/j.imlet.2018.10.006

Russell WMS, Burch RL. *The principles of humane experimental technique*. London: Methuen Ed.; 1959.

Valentini S, Santoro G, Baffetta F, Franceschi S, Paludi M, Brandini E, Gherardini L, Serruto D, Capecchi B. Monocyte-activation test to reliably measure the pyrogenic content of a vaccine: An *in vitro* pyrogen test to overcome *in vivo* limitations. *Vaccine* 2019;37(29):3754-3760. doi:10.1016/j.vaccine.2018.10.082

Vipond C, Findlay L, Feavers I, Care R. Limitations of the rabbit pyrogen test for assessing meningococcal OMV based vaccines. *ALTEX - Alternatives to animal experimentation* 2016;33(1):47-53. doi:10.14573/altex.1509291