Optimization of the Monocyte-Activation-Test for Evaluating Pyrogenicity of Tick-Borne Encephalitis Virus Vaccine

Marilena P. Etna1, Elena Giacomini1, Fabiana Rizzo1, Martina Severa1, Daniela Ricci1, Shahjahan Shaid1, Denis Lambirgis2, Sara Valentini3, Luisa Galli Stampino3, Liliana Alleri2, Andrea Gaggioli2, Christina von Hulotstein1, Ingo Spreitzer1 and Eliana M. Coccia1

1Department of Infectious Diseases, Istituto Superiore di Sanità, Rome, Italy; 2GSK, Wavre, Belgium; 3GSK Vaccines Srl, Siena, Italy; 4Paul Ehrlich Institute, Federal Agency for Sera and Vaccines, Langen, Germany

Abstract

Pyrogen content is a key quality feature that must be checked in all injectable products, including vaccines. Four tests are currently available in the European Pharmacopoeia to monitor pyrogen/endotoxin presence: 1) the Rabbit Pyrogen Test (RPT), 2) the Bacterial Endotoxin Test, 3) the Recombinant Factor C test and the Monocyte Activation Test (MAT). Here, we explored the possibility to replace RPT with the MAT in the quality control of a vaccine against tick-borne encephalitis virus (TBEV). The testing was carried out by using cryopreserved peripheral blood mononuclear cells as cellular source. IL-6 release was selected as readout for the detection of both endotoxin and non-endotoxin contaminants. MAT applicability for pyrogen testing of the TBEV vaccine was assessed through preparatory tests and resulted in the set-up of a very sensitive assay (limit of detection, LOD = 0.04 EU/ml; Sensitivity = 0.1 EU/ml). Both quantitative Method A and semi-quantitative Method B were used for data analysis. Our studies revealed that for vaccine without intrinsic pyrogenicity, as that against TBEV, sensitivity (the lowest endotoxin value of the standard curve) should be used instead of LOD in order to define a stable maximum valid dilution of the product. In conclusion, we describe the challenges of MAT implementation for anti-TBEV vaccine following the current MAT chapter 2.6.30 and propose a re-evaluation of the validity criteria of the Methods A and B, in order to set a semi-quantitative or limit test suitable for those products for which a reference lot comparison analysis is not applicable or favorable.

1 Introduction

Tick-borne encephalitis (TBE) is an illness caused by tick-borne encephalitis virus (TBEV) infection, whose clinical manifestations range from febrile illness to very aggressive downstream neurological symptoms (Ruzek et al., 2019). Three TBEV subtypes, namely the European (TBEV-Eu), the Siberian (TBEV-Sib), or the Far Eastern (TBEV-FE), circulate in forested areas of Europe and northeastern Asia. In addition, two other subtypes - the Baikalian (TBEV-BkI) and the Himalayan (TBEV-Him) - have been recently described.

Few vaccines have been developed using the different TBEV subtypes. In particular, two European vaccines have been produced: FSME-IMMUN (Pfizer, USA), prepared from the Neudoerfl strain of the European subtype (Barrett et al., 2003), and Encepur (GSK), based on the Karlsruhe (K23) strain (Harabacz et al., 1992; Girgsdies and Rosenkranz, 1996). These vaccines have been used for more than 30 years and are highly effective in preventing TBE (Barrett et al., 2003).

The possibility that in the manufacturing processes pyrogenic material may originate from growth of contaminating bacteria or as carry over of an unsuccessful purification step must be avoided and, accordingly, vaccines as well as all parenteral medicines are tested for pyrogens. These fever-inducing substances are derived from gram-negative and gram-positive bacteria (endotoxin, lipotheichoic acid) viruses, fungi, and other sources (Dinarello et al., 1984; Hoffmann et al., 2005), and are related to various pathological conditions, ranging from vascular alteration to shock and death. Thus, to assure a consistent safe profile, the pyrogen content needs to be considered on the basis of the clinical trial lots.

Four tests are currently available in the European Pharmacopoeia (Ph. Eur.) to monitor non-endotoxin/endotoxin presence in pharmaceuticals: 1) the Rabbit Pyrogen Test (RPT, Ph. Eur. 2.6.8); 2) the Bacterial Endotoxin Test (BET, Ph. Eur. 2.6.14), known as the Limulus Amebocyte Lysate (LAL) test; 3) the Recombinant Factor C test (rFC Ph. Eur. 2.6.32) and 4) the Monocyte Activation Test (MAT, Ph. Eur. 2.6.30) (Hartung et al., 2001; Perdono-Morales et al., 2011; Hasiwa et al., 2013).
While the first two assays are based on the use of animals or animal-derived reagents, rFC employs a non-animal derived reagent and MAT uses human whole-blood, peripheral blood mononuclear cells (PBMC) or monocytes cell lines; therefore, both rFC and MAT are in accordance with the ‘3Rs’ principle of Replacement, Reduction and Refinement (Flecknell, 2002; Russell and Burch, 1959) and EU Directive 2010/63/EU concerning the protection of animals. Although both rFC and MAT are not animal-based methods, rFC senses endotoxin pyrogens only, while MAT detects the presence of both endotoxin and non-endotoxin pyrogens (NEPs) (Ph. Eur. 2.6.32 and 2.6.30) via the induction of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6). In this regards, rFC assay has been recently admitted for use as an alternative to the compendial LAL assay (Marius et al., 2020) while, MAT represents an alternative and a more physiological test than the RPT for the detection of endotoxin and NEPs in products for human use, such as vaccines.

The current animal-based methods used for pyrogenicity testing, BET and RPT, display some limits: i) BET detects only the most common pyrogen, endotoxin, and fails to reveal the presence of NEPs (such as peptidoglycan, lipoproteins and bacterial DNA) as well as the presence of specific molecular conformation of lipid A within LPS aggregates necessary to stimulate factor C; ii) both BET (indirect) and RPT (direct) are based on animal use and therefore do not completely reflect the human immune response (Gutsmann et al., 2010; Hasiwa et al., 2013).

In addition, the use of laboratory animals in biomedical research is under discussion for several reasons: i) animal-based quality control tests have a high inherent variability compared to in vitro methods; ii) animal tests and studies are costly and time consuming; iii) the duration of animal test and the number of repeats given the rate of false-positive and false-negative test results can be a bottleneck in the supply of vaccines (Valentini et al., 2019). Thus, it is not conceivable that the availability of vaccines can be restricted due to the limitations of the animal tests.

The amount of mandatory animal use should not be overlooked and poses animal welfare concerns since on a global scale for established vaccines large numbers of laboratory animals are used (De Mattia et al., 2011). Moreover, vaccines are becoming increasingly complex with multiple components along with the development of novel adjuvants designed to evoke the human innate immune response, leading to RPT unsuitability for the testing of these new vaccines (Gutsmann et al., 2010; Vipond et al., 2016). In this context, MAT represents the method of choice for pyrogen testing to overcome these difficulties.

Thus, we investigated the possibility to replace RPT with MAT when testing for pyrogenicity Encepur, a TBEV vaccine, for which the Ph. Eur. prescribes RPT. MAT Method A, quantitative test, and Method B, semi-quantitative test, of the Ph.Eur. chapter 2.6.30 were used and relative results are reported and discussed. In particular, in this case, where a vaccine without intrinsic pyrogenicity was tested and for which the requirement is “not pyrogenic”, it becomes evident that an adaptation of the two method validity criteria was necessary to fulfill at the best Ph. Eur. requirements.

2  Material and methods

Ethics statement
Istituto Superiore di Sanità Review Board approved the present research project (Reference number AOO-03/04/2019 0010821). No informed consent was given since anonymous blood bags were kindly donated by the Blood Transfusion Service and Hematology department of Umberto I Hospital (Rome, Italy). An agreement with the Immunology Unit at Department of Infectious Diseases - Istituto Superiore di Sanità (Rome, Italy), was set in place for picking up blood bags excluded for insufficient volume.

Vaccine samples
All vaccine samples were kindly provided by GSK within the framework of the IMI Project Vac2Vac. The Encepur vaccine is produced using the TBEV-Eu strain K23 that is cultivated on chicken embryo fibroblasts and inactivated with formaline. The excipient matrix possesses physiochemical properties and chemical composition similar to the drug product but does not contain the inactivated TBEV. Encepur for adults (i.e. from 12 years and above) is licensed since 1991. The childhood vaccine is licensed since 1994 for the use from 1 to 11 year old. Encepur is free of preservatives, human serum albumin or other protein-derived stabilizers. One dose for children is a suspension of 0.25 ml containing 0.75 µg of inactivated K23 and 0.15-0.20 mg aluminum hydroxide as adjuvant. One dose for adults is a suspension of 0.5 ml containing 1.5 µg of inactivated K23 and 0.3-0.4 mg aluminum hydroxide as adjuvant.

Pyrogenic stimuli
The USP Reference Standard Endotoxin (TLR4 ligand, 10,000 USP Endotoxin Units per vial) from Lonza (Basel, CH, Switzerland), was used as Reference Standard Endotoxin (RSE). The lyophilized content was reconstituted in LAL Reagent Water (LONZA, Walkersville, MD, USA), according to the manufacturer’s instructions. The stock solution was aliquoted in cryovials (endotoxin E700, 2,000 EU/ml) and stored at -80°C. Dose-response curves with step 4 dilution (starting from 10 EU/ml to 0.00015 EU/ml) and step 2 dilution (starting from 1.6 EU/ml to 0.006 EU/ml) (Fig. S1A1) were initially tested.

Either the synthetic diacylated lipopeptide FSL-1 (TLR2/6 ligand) or the imidazooquinoline compound Resiquimod (R848, TLR7/8 ligand) were both purchased from Invivogen (San Diego, CA) in the vaccine grade formulation and used as NEP stimulants. The lyophilized content of each vial was reconstituted in LAL Reagent Water to obtain a 1 mg/ml stock solution (LONZA, Walkersville, MD, USA), according to the manufacturer’s instructions. For FSL-1 dose-response curves with step 4 dilution (starting from 40 µg/ml to 0.0006 µg/ml) and step 2 dilution (starting from 0.078 µg/ml to 0.0002 µg/ml) (Fig. S1B,C1) were first performed. For R848 dose-response curves with step 4 dilution (starting from 40 µg/µl to 0.0006 µg/µl) and step 2 dilution (starting from 0.9 µg/µl to 0.004 µg/µl) (Fig. S1B,C1) were initially tested.

Isolation of peripheral blood mononuclear cells (PBMC) and cryopreservation

1 doi:10.14573/altex.2002252s
PBMCs were isolated within 4 h from the blood by density gradient centrifugation at 800 g for 30’ at Room Temperature (RT), using Lympholyte-H (Lampire Laboratories, Burlington, ON, Canada). The isolated PBMCs were then resuspended in culture medium RPMI-c (RPMI 1640 w/Hepes, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1X MEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, Life Technologies Italia) and 1% inactivated human AB serum (Sigma-Aldrich, St. Louis, MO, USA). Cells were counted and resuspended in human AB serum supplemented with 10% DMSO (Sigma-Aldrich) and 20–30 x 10^6 PBMCs were dispensed in each cryovial. Cryopreserved cells were frozen at -80°C for at least 24 h and then transferred to a nitrogen tank (-135°C) for long-term storage. At least 10 cryo-preserved cell aliquots were prepared from a minimum of 10 different donors. Only cryo-preserved PBMC aliquots from blood donors negative for HIV1/2, HCV and HBV were stored for their application as cell source.

**Thawing and evaluation of cell viability**

The thawing solution contains Phosphate Buffered Saline (PBS, Lonza), supplemented with 2.5 mM EDTA (Life Technologies) and 20 µg/ml DNase (Sigma-Aldrich). Cryovials removed from the liquid nitrogen tank were transferred to a 37°C water bath and then diluted by adding 1 ml of thawing medium drop-wise directly into each cryovial, then slowly each cell suspension was transferred to a tube pre-filled with warm thawing medium. The cryovials were washed again with 1 ml of thawing medium and cells were centrifuged at 310 g for 10 minutes at RT. The supernatant was discarded and a second centrifugation was performed. The PBMC pellet was resuspended in RPMI-c and viable cells counted by using 0.4% trypan blue. The cell concentration was adjusted to 1.0 x 10^6 ± 20% cells/ml in RPMI-c.

**Cell culture stimulation**

Cell culture stimulation was performed according to method A and B described in the Ph. Eur. chapter 2.6.30. Briefly, two pre-dilutions of the vaccine, RSE and NEPs were performed prior to seed the stimuli in the 96-well plate. Then, step 2 dilutions were performed directly in a flat bottom 96-well cell culture plate (Costar - Corning, NY, USA). In particular, quadruplicates of vaccine dose-response curve from 1:3 to 1:800 were prepared in the 96-well cell culture plate. Then, 100,000 cells were added to each well to a final volume of 200 µl. For the spike, a culture medium containing the spiking dose of RSE or R848 was used instead of the normal one for both the preparation of the vaccine dose-response curve and the plating of PBMC. Culture plates were incubated at 37°C (± 1°C) for 22 ± 1 h in a humidified atmosphere containing 5% CO₂. After incubation of the cells, the supernatant from each well was recovered and cytokine determination was performed.

**Cytokine determination**

As described in MAT chapter of the Ph. Eur. (EDQM, 2017), the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β represent suitable read-outs of monocyte or monocytic cell activation upon stimulation with substances having a pyrogenic activity. Thus, to define the optimal cytokine as read-out of the MAT test in the presence of endotoxin and NEPs, TNF-α, IL-1β and IL-6 release was determined by Cytometric Bead Assay (CBA; BD Biosciences, San Jose, USA). In MAT assay, a modified version of the Duoset IL-6 ELISA kit from R&D Systems (Minneapolis, MN, USA) was used to measure IL-6 production. In particular, the major modification concerned the volume of culture supernatant tested as well as the volume of capture and detection antibody and the incubation time of the substrate.

**Definition of LOD, sensitivity, CLC and MVD**

The limit of the detection (LOD) of the assay was defined as described in Ph. Eur. by identifying in the endotoxin standard curve the concentration corresponding to the cut-off value. The cut-off was calculated as the mean optical density (OD) of 4 replicates of not stimulated cells (blank) + 3 SD of the OD values. LOD for NEPs was likewise determined. During the preparatory testing, standard curves which cover the small dynamic range of MAT response to endotoxin (approximately 1 log10, see Guidance Ph. Eur. 2.6.30.) and NEPs have been defined. As in BET, the lowest LPS concentration which is detected in cell samples from several donors or pools was determined as “sensitivity” of the assay (AS). AS is the lowest or one of the lowest (depending on linear or non-linear setup of the standard curve) standard value close to the beginning of the linear part of the endotoxin or NEPs standard curve.

The contaminant limit concentration (CLC) was determined as the ratio between the threshold of pyrogenic dose per kilogram of body mass (K) and the maximum recommended dose of product per kilogram of body mass (M).

According to the current Ph.Eur guidelines, the maximum valid dilution (MVD) of vaccine is defined as the ratio between CLC and LOD, while, in the proposed modified version of Method B, MVD is calculated as ratio between CLC and sensitivity. In particular, the MVD was calculated by considering the threshold of pyrogenic dose (CLC) assumed for parenteral administration (5 EU/kg) and the maximum recommended dose of product for pediatric administration (0.05 ml/kg) (for details see material and method section).

**PBMC qualification**

The suitability of cells from each donor was evaluated prior to use either in preparatory tests or in the analytical sessions performed with final layouts. Cells recovered after thawing procedure have been monitored for cell viability by trypan blue exclusion staining, by considering only those with viability equal to or higher than 85%, and by evaluating cell response to scalar doses of RSE (from 0.025 to 0.8 EU/ml) in terms of IL-6 production by ELISA. In particular, only PBMC showing a good linear correlation (p < 0.01) among 4 of the chosen RSE doses were utilized for further analyses.

**Test for interfering factors**

To exclude possible interference of the TBEV vaccine with the detection of both endotoxin and NEPs, the vaccine and the excipient matrix were diluted as described above, with parallel samples either spiked (S) or un-spiked (US) with RSE at 0.2 or 0.1 EU/ml or with R848 at 0.3 µg/ml.

Lack of vaccine interference was evaluated by spike recovery. In particular, it was calculated by using the mean values of the endotoxin or non-endotoxin equivalent concentration of the S and US vaccine, according to the equation: %RecoveryMAT = ([S-
US/(spike-in dose) x 100. Dilutions with endotoxin recovery within 50-200% range were considered interference-free. Data are expressed as the mean ± SD of 4 replicates.

**Interference in the detection system**

The IL-6 standard curve was spiked with different vaccine serial dilutions (starting from 1:3 equal to 1 µg/ml of antigen to 1:50 corresponding to 0.0625 µg/ml of antigen, step 2) and tested by ELISA in comparison to the un-spiked IL-6 standard curve. The mean percentage of human IL-6 activity (in terms of OD values) in the presence of each TBEV vaccine dilution was calculated taking into account the results from the un-spiked IL-6 standard curve, which corresponds to 100% of IL-6 activity. There was no interference when the variation of OD was within ± 20 %.

**Validity criteria**

To test TBEV vaccine lot pyrogen level by applying Method A described in Ph. Eur., the following assay validity criteria were used for data generated for each PBMC donor: 1) the OD of not stimulated PBMC (blank) should be below 0.1 OD units; 2) the regression of responses, log-transformed if necessary, on RSE log10 dose shall be statistically significant (p < 0.01); 3) the regression of responses on RSE log10 dose must not deviate significantly from linearity (p > 0.05). [as cited in the Ph. Eur. chapter 2.6.30] (EDQM, 2017).

When Method B was instead applied for testing TBEV vaccine pyrogen level, assay validity for each tested donor was established by considering that: 1) the OD of not stimulated PBMC (blank) should be below 0.1 OD units; 2) the mean response of growing doses of the RSE curve should increase progressively.

All statistical analyses have been conducted according to Ph.Eur. chapter 5.3 by using the statistical analysis software CombiStatsTM (version 5.0) EDQM (Council of Europe, Strasbourg, France). If not otherwise specified, data were expressed as the mean ± SEM.

**Calculation and interpretation of data for the vaccine preparation**

As stated by Ph. Eur. (chapter 2.6.30), for the application of both Method A and Method B, each analytical session was conducted by using cells from 4 different donors and the TBEV vaccine was requested to comply the test with the PBMC of all used donors. When the vaccine passed the test with the cells of 3 of the 4 donors, PBMC from further 4 donors were used. In particular, for Method A, the pyrogen content of each tested vaccine solutions was calculated through the endotoxin dose-response curve in terms of equivalent of endotoxin (eEU/ml) by also applying a correction for the used dilution factor. The preparation was considered to comply with the test when the pyrogen content was less than the CLC. When the current Method B version was applied, the response of the vaccine solution chosen for the pass/fail decision and the dilutions below, all complied with the test if below the LOD while, AS was considered in the proposed modified version of the method. Both current and modified Method B are able to reveal a pyrogen concentration less than the CLC. For both methods, as for the test for interfering factors described above, only vaccine dilutions spiked-in with endotoxin that showed a recovery within 50-200% range fulfilled the requirements.

**Precision study**

As for the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q2(R1), precision should be considered at two levels: repeatability and intermediate precision. For both repeatability and intermediate precision, 3 separate studies were considered, by using the OD values of each of the chosen RSE doses namely 0.1 EU/mL (AS), 0.2 EU/ml (2 X AS, corresponding to spike-in dose) and 0.4 EU/mL (4 X AS). The precision data analyses were performed using variance components, by random models with restricted maximum likelihood estimates (REML). Potential deviations from normal distribution were assessed by Shapiro-Wilk Test (α = 0.05), and, when appropriate the analysis were performed on Natural-Log scale. Dixon’s Test (α = 0.05) was used to exclude outliers within the replicates for each assay. Acceptance criteria for the precision of the method were the following: relative standard deviation (RSD) ≤ 20 % for the repeatability and RSD ≤ 35 % for the intermediate precision. Statistical analysis for the Precision study is performed by IBM SPSS Statistics 25.0.

### 3 Results

**3.1 Comparison of TNF-α, IL-6 and IL-1β as MAT read-outs for the detection of both endotoxin and non-endotoxin contaminants**

To define which cytokine would be the most appropriate read-out in our setting, a simultaneous cytokine measurement was performed on PBMC supernatants stimulated with both endotoxin and non-endotoxin stimuli. In particular, PBMC were treated with RSE triggering TLR4 activation as endotoxin contaminant and with the TLR7/8 agonist R848 or the synthetic TLR2/6 agonist FSL-1 as non-endotoxin contaminants. From our multiplex analysis, IL-6 was chosen as marker of monocyte activation since IL-6 resulted as the most secreted cytokine among those analyzed from PBMC stimulated with different doses of both endotoxin and non-endotoxin contaminants (Fig. 1).

**3.2 Evaluation of cell viability and responsiveness to RSE of cryo-preserved PBMC**

In the attempt to evaluate the stability of PBMC stored in liquid nitrogen, a follow-up study was conducted at 6, 12 and 18 months after cell cryo-preservation by monitoring cell viability as well as cell responsiveness to different doses of RSE. Interestingly, data obtained from cells of 4 different donors demonstrated that after cell thawing at the different times of analysis both the percentage of viable cells and the capacity of PBMC stimulated with 0.2 or 0.4 EU/ml RSE to release IL-6 in culture supernatants remained stable (Tab. 1). These evidences indicated that cryo-preserved PBMC could be used for MAT up to 18 months from their initial storage in liquid nitrogen.
Peripheral blood mononuclear cells (PBMC) were stimulated with RSE (A) [0.2 and 0.4 EU/ml], R848 (B) [0.15 and 0.3 mg/ml], FSL-1 (C) [0.02 and 0.04 ng/ml] or left untreated and TNF-α, IL-6 and IL-1β production tested in culture supernatants by cytometric bead assay. The results shown were mean values ± standard error of the mean of three independent experiments.

Tab. 1: Follow-up study on cell viability and responsiveness to RSE in cryo-preserved PBMC

Stability and responsiveness of cryopreserved peripheral blood mononuclear cells (PBMC) were assayed by testing cell viability and IL-6 release after stimulation with RSE in cells thawed after 6, 12 and 18 months from their storage in liquid nitrogen.

<table>
<thead>
<tr>
<th>Cell viability (%)</th>
<th>RSE 0.2 EU/ml (IL-6 pg/ml)</th>
<th>RSE 0.4 EU/ml (IL-6 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 months</td>
<td>12 months</td>
</tr>
<tr>
<td>PBMC_Donor 1</td>
<td>89.0</td>
<td>93.0</td>
</tr>
<tr>
<td>PBMC_Donor 2</td>
<td>91.2</td>
<td>95.5</td>
</tr>
<tr>
<td>PBMC_Donor 3</td>
<td>95.0</td>
<td>92.0</td>
</tr>
<tr>
<td>PBMC_Donor 4</td>
<td>91.0</td>
<td>93.7</td>
</tr>
</tbody>
</table>

3.3

Setting of MAT conditions for anti-TBEV vaccine

To further define MAT applicability to the TBEV vaccine used in this study and, therefore, to ensure both precision and validity of the test, some parameters and conditions were defined as recommended by Ph. Eur. monograph by performing the four preparatory tests, namely “Assurance of criteria for endotoxin standard curve”, “Interference in the detection system”, “Test for interfering factors” and “Method validation for non-endotoxin contaminants”.
3.4 Assurance of criteria for the endotoxin standard curve

The endotoxin standard curve represents a useful approach to estimate the pyrogenicity of the sample (in EU/ml) by MAT. According to Ph. Eur. requirements, PBMC were stimulated with 4 doses of RSE, namely 0.05 (AS x 0.5), 0.1 (AS), 0.2 (AS x 2) and 0.4 (AS x 4) EU/ml, or were left untreated (blank cells) and ELISA was used to measure IL-6 released in culture supernatants. As shown in Figure 2, the chosen RSE doses properly arranged in a linear fitting. Interestingly, the standard curve correctly met the 2 acceptance
criteria prescribed by Ph. Eur., i.e. the regression of response is statistically significant (p<0.01) as required by both Methods (A and B) and there is no significant deviation from linearity (p>0.05) as needed by Method A only.

3.5 Test for interfering factors
To assure the validity of the test by excluding any interference of the vaccine with the detection of endotoxin as well as to evaluate the trueness of the assay, a test for interfering factors was performed. PBMC were stimulated with 9 serial dilutions of anti-TBEV vaccine alone or spiked-in with 0.2 EU/ml of RSE, corresponding to 2 x AS and, at the end of the incubation, IL-6 ELISA was performed to determine the amount of corresponding equivalent of EU/ml (eEU/ml). In agreement with the Ph. Eur. 2.6.30 and based on our results, vaccine dilution starting from 1:12.5 could be considered interference-free, since the RSE recovery was within the permitted range of 50-200% (Fig. 3A). As expected, the interference observed at higher doses of vaccine was due to the excipient matrix that, when used in the test for interfering factors, allowed RSE recovery at the same dilutions of the vaccine (Fig. 3B).

Fig. 3: Recovery of RSE at different doses of ENCEPUR vaccine and excipient matrix
Peripheral blood mononuclear cells (PBMC) were stimulated with nine different doses of Encepur vaccine or excipient solution spiked-in with a fixed dose of RSE (0.2 EU/ml) or R848 (0.3 µg/ml). Recovery of RSE or R-848 was measured by IL-6 release in terms of equivalent of endotoxin unit per milliliter (eEU/ml) or equivalent of R848 microgram per milliliter (eµg/ml) respectively, by ELISA. The range of valid eEU/ml or eµg/ml values (50-200% of the spiked dose of RSE or R848) is indicated by red dashed lines. Dots represent single recovery values obtained from 5 different donors for the spike of RSE in the Encepur vaccine (A) or in the excipient matrix (B) and 4 different donors when R848 was spiked-in into Encepur vaccine (C) or excipient matrix (D).

3.6 Method validation for non-endotoxin contaminants
The main reason for considering MAT a reliable in-vitro replacement of RPT is the unique capacity of this assay to detect NEPs in addition to bacterial endotoxin. The MAT setting was validated with the two TLR agonists FSL-1 and R848 (Fig. 2). For both NEPs, a dose response curve showed a linear portion displaying a statistically significant regression of response (p<0.01) and a not significant deviation from linearity (p>0.05) (Fig. 2).

In addition, the interference of TBEV vaccine with the detection of non-endotoxin contaminants was evaluated in the test for interfering factors. PBMC were treated with 9 serial dilutions of anti-TBEV vaccine alone or spiked-in with 0.3 µg/ml of R848, corresponding to 2 x AS. R848 recovery percentage was determined as previously shown for the RSE (Fig. 3C) and fell within the range of 50-200% starting from 1:50 TBEV vaccine dilution, although, when excipient matrix was used instead of the vaccine, the recovery started from 1:100 (Fig. 3D). Based on these data, 1:100 vaccine dilution was chosen to avoid TBEV vaccine interference with the NEPs' detection.

3.7 Interference of TBEV vaccine in the detection system
In addition, vaccine interference with the detection system for the chosen read-out must be also evaluated. To this aim, the 5 highest vaccine doses were added to IL-6 standard curve. Accordingly to Ph. Eur. recommendations, all the detected OD variations due to vaccine addition, at all the analyzed concentrations, fell within +/- 20% of the OD detected with the recombiant protein alone (Tab. 2) indicating that the vaccine does not interfere with the ELISA detection system.

3.8 Determination of LOD, sensitivity, CLC and MVD
To define the proper conditions for the TBEV vaccine specific MAT optimization, we determined the LOD as well as the assay sensitivity (AS) for the measurement of both endotoxin and non-endotoxin pyrogenic substances (Tab. 3). The obtained values underlined that the assay developed so far is a highly sensitive test, since it is able to detect up to 0.04 EU/ml [10 times lower than the most sensitive RPT setup (with 10 ml injection volume per kg of body weight)] and to quantify up to around 0.1 EU/ml of RSE (Tab. 3). Similarly, very little amounts of both R848 and FSL-1 can be detected in our experimental setting as shown by LOD and AS values inserted in Table 3.

By considering the parameters for parenteral and pediatric administration (see material and method section), the MVD for Encepur vaccine in the MAT assay was defined to be 1:2700 (Scheme 1). However, since the AS is closer to the linear part of the RSE curve, where a more precise estimation of pyrogenicity in eEU/ml is possible (Table 4A, 4B and Fig. S1) a potential alternative for calculating MVD might be to replace LOD with sensitivity. By using sensitivity, the proposed new MVD for the TBEV vaccine is 1:1000 (Scheme 1).

Tab. 2: Evaluation of Encepur interference with the ELISA procedure

Five doses of Encepur (V1= 1 µg/ml [1:3], V2= 0.5 µg/ml [1:6], V3= 0.25 µg/ml [1:12.5], V4= 0.125 µg/ml [1:25] and V5= 0.0625 µg/ml [1:50]) were added to the IL-6 standard curve. The interference of Encepur with the ELISA procedure was evaluated by considering optical density values of the IL-6 standard alone or in combination with the vaccine.

<table>
<thead>
<tr>
<th>STANDARD</th>
<th>STANDARD+V1</th>
<th>STANDARD+V2</th>
<th>STANDARD+V3</th>
<th>STANDARD+V4</th>
<th>STANDARD+V5</th>
</tr>
</thead>
<tbody>
<tr>
<td>O.D.</td>
<td>Interference (%)</td>
<td>O.D.</td>
<td>Interference (%)</td>
<td>O.D.</td>
<td>Interference (%)</td>
</tr>
<tr>
<td>600</td>
<td>2.629</td>
<td>2.549</td>
<td>-3.4%</td>
<td>2.507</td>
<td>-4.6%</td>
</tr>
<tr>
<td>300</td>
<td>1.788</td>
<td>1.770</td>
<td>-1.0%</td>
<td>1.818</td>
<td>+1.6%</td>
</tr>
<tr>
<td>150</td>
<td>1.095</td>
<td>1.061</td>
<td>-3.1%</td>
<td>1.024</td>
<td>-6.4%</td>
</tr>
<tr>
<td>75</td>
<td>0.593</td>
<td>0.555</td>
<td>-6.4%</td>
<td>0.565</td>
<td>-4.7%</td>
</tr>
<tr>
<td>37.5</td>
<td>0.294</td>
<td>0.240</td>
<td>-18.3%</td>
<td>0.291</td>
<td>-1.0%</td>
</tr>
<tr>
<td>18.8</td>
<td>0.140</td>
<td>0.131</td>
<td>-6.4%</td>
<td>0.130</td>
<td>-7.1%</td>
</tr>
<tr>
<td>9.38</td>
<td>0.076</td>
<td>0.073</td>
<td>-3.9%</td>
<td>0.062</td>
<td>-18.4%</td>
</tr>
</tbody>
</table>

Tab. 3: Definition of assay LOD and sensitivity

Limit of the Encepur-optimized MAT assay in detecting (LOD) endotoxin (RSE) as well as non-endotoxin (R848 and FSL-1) contaminants was calculated according to Ph. Eur. recommendations. Assay sensitivity (AS) has been determined as the lowest (limit test) or low concentration of endotoxin (RSE) or non-endotoxin (R848 and FSL-1)-standard close to the beginning (Limit of Quantification) of the linear part of the standard curve.

<table>
<thead>
<tr>
<th>TLR agonist</th>
<th>LOD</th>
<th>LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSE (EU/ml)</td>
<td>0.040</td>
<td>0.100</td>
</tr>
<tr>
<td>R-848 (mg/ml)</td>
<td>0.050</td>
<td>0.150</td>
</tr>
<tr>
<td>FSL-1 (mg/ml)</td>
<td>0.003</td>
<td>0.009</td>
</tr>
</tbody>
</table>

3.9 Application of Method A
Once that all the MAT conditions were established, the vaccine was tested by using Method A. This method is a quantitative method, which involves the comparison of 3 doses of the preparation being examined (1:100, 1:200, 1:400) in quadruplicate, with a standard endotoxin curve in the range of 0.025-0.8 EU/ml. In the plate layout prepared for testing a single vaccine lot, positive control samples spiked with 0.2 EU/ml of RSE (corresponding to the middle dose of the endotoxin standard curve) were included (Scheme 2). Two different analysts performed the assay in a single analytical session and eEU/ml for each vaccine dose, alone or combined with RSE, were determined by using the linear part of endotoxin standard curve (Fig. 4). RSE recovery, regression of response and deviation from linearity were calculated as previously described for preparatory testing.

By applying method A, the amounts of pyrogens detected in the vaccine resulted to be below the LOD thus, confirming the low levels of pyrogens inherently present in this vaccine. Also the recovery of RSE fell within the range of 50-200% for each of the two analysts performing the assay. However, although regression of response met the validity criteria, a significant deviation from linearity was achieved by both analysts and, thus, the test resulted not valid (Fig. 4). Of note, this unsuccessful result was not due to vaccine response in terms of pyrogen content, but to the unachieved fulfillment of the criteria for the endotoxin standard curve.

3.10 Application of Method B
Method B was then considered as a possible alternative for data analysis of products, such as the vaccine used in this study, whose responses induced after cell stimulation were not parallel to dilutions of standard endotoxin. Accordingly, a plate layout containing 4 repetitions of 3 vaccine doses (1:100, 1:200 and 1:400) alone or spiked with 0.1 EU/ml of RSE (2 x LOD) and a 5 points dose response curve of RSE, ranging from 0.025 to 0.4 EU/ml, was conceived and is shown in scheme 3. Even if the vaccine pyrogen level resulted
Safety of injectable medicines and biologicals, in terms of pyrogen content, has been historically tested by using the RPT, which, as many other prescribed pharmacopoeia safety tests, was developed at a time when adverse events due to the test specific contaminants to be below the LOD, no recovery of RSE was obtained when a spike of 2 x LOD was used for preparing endotoxin-contaminated samples, as indicated in Ph. Eur., thus making the assay not valid (Fig. 5A). However, since AS is the first value of the RSE curve, allowing reliable evaluation, the substitution of LOD with AS for both the definition of RSE spike-in dose and for the threshold of the pyrogen content, made the semi-quantitative Method B completely and successfully applicable for pyrogen testing of product with low or no inherent pyrogen content (Fig. 5B). Indeed, the TBEV vaccine pyrogen level resulted to be below the AS and, when an amount of RSE equal to 2 x AS (0.2 EU/ml), was spiked-in in the vaccine, the endotoxin recovery fell within the range of 50-200 %, thus making the test valid (Fig. 5B). According to the guidelines for the validation of analytical methods (ICH-Q2B), the semi-quantitative method B with the proposed modifications was validated by applying the method on three different Encepur lots (data not shown).

3.11 Precision study

Repeatability of the method has been accurately assessed by evaluating the variability of the 4 replicates in the same plate (“Within assay” precision) in a Variance Components model considering Analyst and Day as random factors (contributors to the “Between assay” precision). Two different analysts performed the tests in 5 different days, in the same laboratory and on cell samples from the same donor for a total of 10 assays. As shown in table 4A, the repeatability of the assay proportionally improved with the increment of the RSE doses, namely 0.1, 0.2, 0.4 EU/ml, as evident from the total RSD equal to 19.8, 6.1 and 4.3 % respectively.

The intermediate precision (IP) (total variability) of the method was calculated on 20 sessions, with PBMC samples from 8 different donors, performed by 2 different analysts (Tab. 4B). It has been evaluated by considering Analyst, PBMC Donor and Assay (i.e. analytical variability that accounts for Within and Between assay components) as random factors. As expected, at the middle dose of the endotoxin standard curve (0.2 EU/ml), where the acceptance criteria for precision were foreseen (i.e. 6.1 % for repeatability and 20.3% for intermediate precision), the contribution of the donors resulted to be the biggest portion (equal to 68 %; Tab. 4B) of the total variability, while contribution of the analysts resulted to be ≤ 3%, denoting a high consistency of the two operators’ performance.

On the other hand, the contribution of donors and assay to the total variability became comparable at 0.4 EU/ml, where precision study indicated that the assay is very precise (RSD = 11 %; Tab. 4B). Interestingly, the precision of the method increases linearly with the increment of RSE dose as evident from the decrement of the RSD (Tab. 4A, 4B and Fig. S1). Overall, this method showed a satisfactory precision.

4 Discussion

Safety of injectable medicines and biologicals, in terms of pyrogen content, has been historically tested by using the RPT, which, as many other prescribed pharmacopoeia safety tests, was developed at a time when adverse events due to the test specific contaminants...
Peripheral blood mononuclear cells (PBMC) were stimulated with RSE (R₁ = 0.05, R₂ = 0.1, R₃ = 0.2 and R₄ = 0.4 EU/ml) or Encepur vaccine (1:100, 1:200 and 1:400) according to plate layout presented in scheme 2 and optical density (OD) values were measured by IL-6 ELISA. Statistical analysis was conducted by using Combistats (see table below graphs). Results obtained from experimental sessions performed by two different analysts on PBMC of the same donor are shown. Squares indicated OD obtained by stimulation with Encepur alone, while triangles, OD derived by PBMC treated with vaccine spiked-in with 0.2 EU/ml of RSE. Numbers close to triangles, indicated the percentage of RSE recovery. A representative experiment out of three experiments yielding similar results is shown.

![Method A application](chart-image)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>8.8429</td>
<td>8.8429</td>
<td>90.793</td>
<td>0.000 (***)</td>
</tr>
<tr>
<td>Non-linearity</td>
<td>2</td>
<td>0.3553</td>
<td>0.1776</td>
<td>1.872</td>
<td>0.179</td>
</tr>
<tr>
<td>Residual error</td>
<td>12</td>
<td>1.4119</td>
<td>0.0805902</td>
<td>0.000 (***)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>10.4749</td>
<td>0.89429</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**VALID**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>3.6772</td>
<td>3.6772</td>
<td>349.23</td>
<td>0.000 (***)</td>
</tr>
<tr>
<td>Non-linearity</td>
<td>2</td>
<td>0.19209</td>
<td>0.096045</td>
<td>1.072</td>
<td>0.365</td>
</tr>
<tr>
<td>Residual error</td>
<td>12</td>
<td>0.28472</td>
<td>0.0237002</td>
<td>0.000 (***)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>10.6593</td>
<td>0.66664</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOT VALID**

- RSE
- ENCEPUR
- ENCEPUR + RSE

were frequent. However, the constant progress in production technologies, quality control and quality management, all driven by corrective and preventive actions, make these contaminations extremely rare.

According to European legislation (EU Directive 2010/63/EU) animal tests were and are to be questioned. To this aim, the EU national ministries and 3R-Grants employed several economic efforts which resulted in the validation of the MAT by ECVAM (European Center for the Validation of Alternative Methods) in 2005 (Hoffmann et al., 2005) and implementation of the General Chapter 2.6.30 “Monocyte Activation Tests” into the Ph. Eur. in 2010.

The idea about MAT, previously called In-vitro Pyrogen Test (IPT), was born in the last two decades when the basic mechanism of fever induction was revealed and the cytokine detection by ELISA became a common tool in laboratories (Hartung et al., 2001; Gaines Das et al., 2004; Dinarello, 2004; Schindler et al., 2009). Nowadays, MAT has been successfully implemented only for an outer membrane vesicle-based meningococcal b vaccine, an intrinsically pyrogenic product (Vipond et al., 2019; Valentini et al., 2019), thus avoiding the high rate of false positive results obtained by using RPT. Those frequent RPT-false positives were probably due to the necessity to perform high dilutions of the vaccine before intravenous administration in rabbit, and the LAL limitation in detecting NEPs (Vipond et al., 2016).

Another successful MAT application for human bacterial vaccine is represented by the Shigella sonnei vaccine composed by outer membrane particles derived from genetically modified bacteria to produce penta-acylated LPS with reduced endotoxicity, called Generalized Modules for Membrane Antigens (GMMA). Both MAT and RPT provided similar data showing a lower pyrogenic activity of GMMA respect to modified LPS (Gerke et al., 2015).
Peripheral blood mononuclear cells (PBMC) were stimulated with RSE (R\textsubscript{1} = 0.025, R\textsubscript{2} = 0.05, R\textsubscript{3} = 0.1, R\textsubscript{4} = 0.2 and R\textsubscript{5} = 0.4 EU/ml) or Encepur vaccine (1:100, 1:200 and 1:400) according to plate layout presented in scheme 3 and optical density (OD) value were measured by IL-6 ELISA. Results obtained from experimental session performed by two different analysts on PBMC of the same donor are shown. Squares indicated OD obtained by stimulation with Encepur alone, while triangles represent OD values from PBMC treated with vaccine spiked-in with 0.1 (A) or 0.2 (B) EU/ml of RSE. Numbers close to triangles, indicated the percentage of RSE recovery. Threshold of pyrogenic dose was fixed equal to the assay limit of detection (LOD) as described in Ph.Eur. (A) or equal to the assay sensitivity (AS) (B) in the proposed modified version of Method B. A representative experiment out of three experiments yielding similar results is shown.

Moving from bacterial vaccines to viral ones, de Mattos and colleagues recently showed the applicability of MAT in the quality control of live attenuated yellow fever vaccine, with no intrinsic pyrogenicity, grown in chicken embryos (de Mattos et al., 2018). This study demonstrated the correlation between MAT and LAL in terms of LPS recovery in spiked batches and the confirmation of the absence of pyrogens in the commercial products.

In this study, a vaccine against TBEV virus was tested by MAT in accordance with the Ph. Eur. Method A and then Method B. After the product-specific optimization, both methods showed some limitations in their applicability. In particular, the development of an assay with high sensitivity is mandatory to test an intrinsically non-pyrogenic product. Here, human PBMC and IL-6 quantification were selected to initially perform the preparatory tests, recommended by Ph. Eur. to ensure the validity of the test (EDQM, 2017), and then the final assay.

Indeed, given the expression of wide receptor repertoire for microbial products, PBMC constitute a useful cell platform to reveal the presence of unknown pyrogens, that may derive during the manufacturing processes from the growth of contaminating microbes or as carry-over of an unsuccessful purification step, as recently demonstrated in comparative studies performed by different laboratories (Vipond et al., 2019; Gaines Das et al., 2004; Taktak et al., 1991). Furthermore, among the pro-inflammatory cytokines induced in response to pyrogen sensing and used as possible readout, IL-6, unlike IL-1\textbeta and TNF-\alpha, is totally released in culture medium (Hasiwa et al., 2013), thus providing a robust measure of pyrogen content.

In addition to both endotoxin and NEPs detection, the possible interference of the vaccine with the technique clearly demonstrated that 1:100, corresponding to 0.03 µg/ml of the product, is the first suitable vaccine dilution to be included in the final plate layout. Of note, the interference observed with vaccine dose higher than 0.03 µg/ml was entirely attributed to the excipient matrix.

However, some difficulties were experienced in the choice and applicability of the method of analysis. The MVD calculation for Methods A and B is based on the sensitivity of the chosen MAT setup. The General Chapter 2.6.30 was written by having in mind the nomenclature of BET (Ph. Eur. chapter 2.6.14) thus, using terms like MVD, sensitivity, CLC [equal to the endotoxin limit
concentration (ELC)). From our point of view the sensitivity of the BET-Lysate (λ) was mistranslated to LOD in the MAT chapter. But, according to the chapter 2.6.14, λ is the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques. Our data strongly support the possibility to replace LOD by AS for the calculation of the MVD and for spiking in Method B since AS is a real (not calculated) part of the standard curve (typically the lowest value for the limit test and one of the lowest values for the semi-quantitative approach using a non-linear fit like 5P-sigmoidal). Even for the limit-test version of Method B reliable quantification is necessary for the calculation of the spike recovery. The replacing of LOD, that is variable from day to day, with AS, confirmed in every valid experiment, is the basis for a stable MVD calculation.

For the calculation of the CLC (or ELC in the BET), typically the application of a specific K-value (threshold pyrogenic dose per kg, m² or per eye (intravitreal application)) is needed, whose value is defined in exactly the same way in the chapter 5.1.10 (Guidelines for using the test for bacterial endotoxins) and in the revised general chapter 2.6.30 (Monocyte-activation test). Nevertheless, for products like vaccines that are frequently administered intramuscularly in low volume, there is no K-value specified in the chapter 2.6.30. To overcome the issue of missing K-value, a threshold K-value can be scientifically deduced as done in the RPT for testing TBEV vaccine pyrogenicity, namely one human dose of vaccine/kg body weight.

The current Method A requests a parallel behavior of the product dilutions versus the RSE standard curve, and, consequently, a linear response of the cell system. Accordingly, the curve linearity as assay validity criteria is critical, especially in the presence of a NEP or an intrinsic pro-inflammatory product and, therefore, this requirement is very unlikely to be constantly fulfilled. Indeed, even if in this study cryopreserved PBMC, well-characterized in terms of cell viability and RSE responsiveness, were used, the standard curve occasionally failed to fulfill the linearity criterion, thus implying a not valid test result notwithstanding test sample data accomplished the product-specific limit of pyrogen contents (CLC) and the recovery of endotoxin.

In conclusion, our data provide evidences that the pyrogen level of the vaccine Encepur can be established by MAT with a satisfactory precision as evaluated by repeatability and intermediate precision of the method. However, it is necessary to re-evaluate the restriction of curve linearity with regards to the dilution range. This aspect will not negatively affect the already validated assays showing robust linearity. In addition, if AS will be used instead of LOD, Methods A and B would converge into a semi-quantitative or limit-test. This could be established for all of those products that are not intrinsically pyrogenic, that need to be tested at a range of dilutions that includes minimum valid dilution and for which the reference lot comparison test (Method C) seems excessive or not suitable. As a next step a broader applicability should be discussed to reflect the behavior of further intrinsically not pyrogenic biologicals using metastudies and surveys.

References


Vipond, C., Sutherland, J., Nordgren, K. et al. (2019). Development and validation of a monocyte activation test for the control/safety testing of an omv-based meningococcal b vaccine. Vaccine 37, 3747-3753. doi:10.1016/j.vaccine.2018.06.038

**Conflict of interest**

All authors have declared the following interests: SS, DL, SV, LGS, LA, RC, and GV are employees of the GSK group of companies. DL reports ownership of GSK shares and/or restricted GSK shares. LGS is listed as an inventor on patents owned by the GSK group of companies. MPE, EG, FR, MS, DR, AG, CvH, IS and EMC report no conflict of interest.

**Author contributions**

MPE, EMC, SV, LA were involved in the conception and design of the study. MPE, EG, FR, MS and DR acquired the data. MPE, EG, EMC, AG, CvH, IS, LGS, SV and LA analyzed and assessed the results. MPE, EMC, IS, CvH, SS, LGS, SV and LA were involved in methods selection. All authors were involved in drafting the manuscript or critically revising it for important intellectual content. All authors had full access to the data and approved the final manuscript.

**Acknowledgements**

This study was granted by VAC2VAC project funded from the Innovative Medicines Initiative 2 Joint undertaking under grant agreement N-115924. We acknowledge Dr. Patrizio Pezzotti (Istituto Superiore di Sanità) for his helpful contribution in the evaluation of linearity criteria for the standard curve. We also thank Mafalda Terrosi, Monica Rinaldi, Giovanni Vitali, Raffaella Cecchi (GSK Vaccine Srl, Siena, Italy), Charline Hoebreck (GSK Wavre, Belgium) and Hilde Depraetere (European Vaccine Initiative, Heidelberg, Germany) for providing helpful discussion and technical support. Encepur is a trademark of the GSK group of companies.