Research Article

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

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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: the rabbit pyrogen test (RPT), the bacterial endotoxin test, the recombinant factor C test, and the monocyte activation test (MAT). Here, we explored the possibility to replace the RPT with the MAT in the quality control of a vaccine against tick-borne encephalitis virus (TBEV). The testing was carried out using cryopreserved peripheral blood mononuclear cells as cell 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 establishment 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 a vaccine without intrinsic pyrogenicity, such as that against TBEV, sensitivity (the lowest endotoxin value of the standard curve) should be used instead of LOD 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 Ph. Eur. chapter 2.6.30 and propose a re-evaluation of the validity criteria of 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 highly aggressive downstream neurological symptoms (Ruzek et al., 2019). Three TBEV subtypes, namely the European (TBEV-Eu), the Siberian (TBEV-Sib), and the Far Eastern (TBEV-FE), circulate in forested areas of Europe and northeastern Asia. In addition, two other subtypes – the Baikalian (TBEV-Bkl) 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 pro-

duced: FSME-IMMUN (Pfizer, USA), prepared from the Neu-doerfl 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).

Contamination of the product during the manufacturing process with pyrogenic material, which 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 other parenteral medicines are tested for pyrogens. These fever-inducing substances are derived from Gram-negative and Gram-positive bacteria (endotoxin, lipoteichoic acid), viruses,

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fungi and other sources (Dinarelli 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 safety profile, the pyrogen content needs to meet the specification authorized on the basis of the clinical trial lots.

Four tests are currently included in the European Pharmacopeia (Ph. Eur.) to monitor endotoxin/non-endotoxin contamination 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* amoebocyte 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; Perdomo-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 monocytic cell lines; therefore, both rFC and MAT comply 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 used for scientific purposes.

Like the BET, the 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, EDQM, 2017) via measuring the induction of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β) or interleukin 6 (IL-6). In this regard, the rFC assay has been recently admitted for use as an alternative to the compendial LAL assay (Marius et al., 2020), while the MAT represents an alternative to, and a more physiological test than, the RPT for the detection of endotoxin and NEPs in products for human use, such as vaccines.

The animal-based methods used for pyrogenicity testing, i.e., 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 conformations 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 tests 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 problematic that the availability of vaccines can be restricted due to the limitations of the animal tests.

The amount of mandatory animal use poses animal welfare concerns and should not be overlooked. On a global scale, large numbers of laboratory animals are used for established vaccines (De Mattia et al., 2011). Moreover, vaccines are becoming increasingly complex, containing multiple components and novel

adjuvants designed to evoke the human innate immune response, leading to the RPT being unsuitable for testing these new vaccines (Gutsmann et al., 2010; Vipond et al., 2016). In this context, MAT represents the method of choice to overcome these difficulties for pyrogen testing.

Thus, we investigated the possibility to replace RPT with MAT for pyrogenicity testing of Encepur, a TBEV vaccine, for which the Ph. Eur. currently prescribes RPT. MAT Method A, quantitative test, and Method B, semi-quantitative test, of Ph. Eur. chapter 2.6.30 (EDQM, 2017) were used. In particular, in this case, where a vaccine without intrinsic pyrogenicity is tested and for which the requirement is “not pyrogenic”, it became evident that an adaptation of the two methods’ validity criteria is necessary to optimally fulfill the Ph. Eur. requirements.

2 Materials and methods

Ethics statement

Istituto Superiore di Sanità Review Board approved the present research project (Reference number AOO-ISS 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 from use owing to insufficient volume.

Vaccine samples

All vaccine samples were kindly provided by GSK within the framework of the IMI Project Vac2Vac. The Encepur vaccine is manufactured using the TBEV-Eu strain K23, which is cultivated on chicken embryo fibroblasts and inactivated with formalin. The excipient matrix possesses physicochemical properties and a chemical composition similar to the drug product but does not contain the inactivated TBEV. Encepur for adults (i.e., 12 years and above) has been licensed since 1991. The vaccine for children has been licensed since 1994 for use in 1 to 11-year-olds. 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 μ g 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 μ g 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 tested initially.

Either the synthetic diacylated lipoprotein FSL-1 (TLR2/6 ligand) or the imidazoquinoline compound resiquimod (R848, TLR7/8 ligand), both purchased from Invivogen (San Diego, CA) in vaccine grade formulation, were 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 ng/mL to 0.0006 ng/mL) and step 2 dilution (starting from 0.078 ng/mL to 0.0002 ng/mL) (Fig. S1B,C¹) were first performed. For R848, dose-response curves with step 4 dilution (starting from 40 µg/mL to 0.0006 µg/mL) and step 2 dilution (starting from 0.9 µg/mL to 0.004 µg/mL) (Fig. S1B,C¹) were tested initially.

Isolation of peripheral blood mononuclear cells (PBMC) and cryopreservation

PBMC were isolated within 4 h of blood withdrawal by density gradient centrifugation at 800 g for 30' at room temperature (RT) using Lympholite-H (Cedarlane Laboratories, Burlington, ON, Canada). The isolated PBMC 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 × 10⁶ PBMC 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 cryopreserved cell aliquots each were prepared from a minimum of 10 different donors. Only cryopreserved PBMC aliquots from blood donors negative for HIV1/2, HCV and HBV were stored for use as cell source.

Thawing and evaluation of cell viability

Cryovials removed from the liquid nitrogen tank were transferred to a 37°C water bath and then diluted by adding 1 mL thawing medium (phosphate-buffered saline (PBS, Lonza) supplemented with 2.5 mM EDTA (Life Technologies) and 20 µg/mL DNase (Sigma-Aldrich)) drop-wise directly into each cryovial. Then, each cell suspension was transferred slowly to a tube pre-filled with warm thawing medium. The cryovials were washed again with 1 mL thawing medium, and cells were centrifuged at 310 g for 10 min at RT. The supernatant was discarded and a second centrifugation was performed. The PBMC pellet was resuspended in RPMI-c, and viable cells were counted using 0.4% trypan blue. The cell concentration was adjusted to 1.0 × 10⁶ ±20% cells/mL in RPMI-c.

Cell culture stimulation

Cell culture stimulation was performed according to Methods A and B as described in Ph. Eur. chapter 2.6.30 (EDQM, 2017). Briefly, two pre-dilutions of the vaccine, RSE and NEPs were

performed prior to seeding 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). Quadruplicates of the 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, culture medium containing the spiking dose of RSE or R848 was used 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 Ph. Eur. 2.6.30 (EDQM, 2017), the pro-inflammatory cytokines TNF-α, IL-6 and IL-1 β represent suitable read-outs of monocytic cell activation upon stimulation with substances having 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). Subsequently, a modified version of the Duoset IL-6 ELISA kit from R&D Systems (Minneapolis, MN, USA) was used to measure IL-6 released in the MAT assay. In particular, the major modification concerned the volume of culture supernatant tested as well as the volumes of capture and detection antibody and the incubation time of the substrate.

Definition of LOD, AS, CLC and MVD

The limit of detection (LOD) of the assay was defined as described in Ph. Eur. by identifying the concentration corresponding to the cut-off value in the endotoxin standard curve. The cut-off was calculated as the mean optical density (OD) of 4 replicates of non-stimulated cells (blank) + 3 × SD of the OD values. The LOD for NEPs was determined likewise.

During preparatory testing, standard curves that cover the small dynamic range of the MAT response to endotoxin (approximately 1 log₁₀, see Guidance Ph. Eur. 2.6.30, EDQM, 2017) and NEPs were defined. As in the BET, the lowest LPS concentration detected in cell samples from several donors or pools was determined as assay sensitivity (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 NEP 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 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 AS. In particular, the MVD was calculated by considering the threshold of pyrogenic dose (CLC) assumed for parenteral administration (5 EU/kg)

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and the maximum recommended dose of product for pediatric administration (0.05 mL/kg).

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 the thawing procedure were assessed for cell viability by trypan blue exclusion staining, considering only those with viability $\geq 85\%$, 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. 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 unspiked (US) with 0.2 or 0.1 EU/mL RSE or with 0.3 $\mu\text{g/mL}$ R848. Lack of vaccine interference was evaluated by spike recovery. In particular, it was calculated using the mean values of the endotoxin or non-endotoxin equivalent concentration of the S and US vaccine according to the equation: $\% \text{Recovery}_{\text{MAT}} = ([S - US] / \text{spike-in dose}) \times 100$. Dilutions with endotoxin recovery within the range of 50-200% were considered interference-free. Data are expressed as the mean $\pm$ 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 $\mu\text{g/mL}$ of antigen to 1:50 corresponding to 0.0625 $\mu\text{g/mL}$ of antigen, step 2) and tested by ELISA in comparison to the unspiked 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 unspiked IL-6 standard curve, which corresponds to 100% IL-6 activity. There was deemed to be no interference when the variation of OD was within $\pm 20\%$.

Validity criteria

To test TBEV vaccine lot pyrogen level by applying Method A as described in Ph. Eur., the following assay validity criteria were used for data generated for each PBMC donor: 1) the OD of non-stimulated PBMC (blank) should be below 0.1 OD units; 2) the regression of responses, log-transformed if necessary, on RSE $\log_{10}$ dose shall be statistically significant ($p < 0.01$); 3) the regression of responses on RSE $\log_{10}$ dose must not deviate significantly from linearity ($p > 0.05$) (as cited in Ph. Eur. chapter 2.6.30, EDQM, 2017).

When Method B was applied for testing TBEV vaccine pyrogen level, assay validity for each tested donor was established by considering that: 1) the OD of non-stimulated PBMC (blank) should be below 0.1 OD units; 2) the mean response to increasing doses of RSE should increase progressively.

All statistical analyses were conducted according to Ph. Eur. chapter 5.3 using the statistical analysis software Combistats™

(version 5.0 EDQM/Council of Europe, Strasbourg, France). If not otherwise specified, data were expressed as the mean $\pm$ SEM.

Calculation and interpretation of data for the vaccine preparation

As stated in Ph. Eur. (chapter 2.6.30, EDQM, 2017) for the application of both Method A and Method B, each analytical session was conducted using cells from 4 different donors, and the TBEV vaccine was required to pass the test with the PBMC of all donors used. If the vaccine passed the test with the cells of only 3 of the 4 donors, PBMC from a further 4 donors were used. In particular, for Method A, the pyrogen content of each tested vaccine solution was calculated through the endotoxin dose-response curve in terms of equivalent of endotoxin (eEU/mL) by 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 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 a range of 50-200% fulfilled the requirements.

Precision study

According to 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 (IP). For both repeatability and IP, 3 separate studies were considered based on the OD values of each of the chosen RSE doses, namely 0.1 EU/mL (AS), 0.2 EU/mL ($2 \times$ AS, corresponding to spike-in dose) and 0.4 EU/mL ($4 \times$ 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 ($\alpha = 0.05$), and, when appropriate, the analyses were performed on natural-log scale. Dixon's Test ($\alpha = 0.05$) 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) $\leq 20\%$ for the repeatability and RSD $\leq 35\%$ for the IP. Statistical analysis for the precision study was performed using IBM SPSS Statistics 25.0.

3 Results

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

To define which cytokine is 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,

Fig. 1: Pro-inflammatory cytokine release in PBMC cultures stimulated with endotoxin and non-endotoxin contaminants
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-1 β production was assessed in culture supernatants by cytometric bead assay. The results shown are mean values $\pm$ SEM of three independent experiments.

which triggers 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 was the most highly 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 cryopreserved PBMC

To evaluate the stability of PBMC stored in liquid nitrogen, a follow-up study was conducted at 6, 12 and 18 months after cell cryopreservation to monitor cell viability as well as cell responsiveness to different doses of RSE. Data obtained from cells of 4 different donors demonstrated that after thawing at the different times 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 results indicate that cryopreserved PBMC can be used for MAT for up to 18 months after their initial storage in liquid nitrogen.

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 in the Ph. Eur. monograph by performing the four preparatory tests, namely “Assurance of criteria for endotoxin standard curve”, “Test for interfering factors”, “Method validation for non-endotoxin contaminants”, and “Interference in the detection system”.

Assurance of criteria for the endotoxin standard curve

The endotoxin standard curve represents a useful approach to estimate the pyrogenicity of the sample (in eEU/mL) by MAT. According to Ph. Eur. requirements, PBMC were stimulated with 4 doses of RSE, namely 0.05 (AS $\times$ 0.5), 0.1 (AS), 0.2 (AS $\times$ 2) and 0.4 (AS $\times$ 4) EU/mL, or were left untreated (blank), and ELISA was used to measure IL-6 released into culture supernatants. As shown in Figure 2, the chosen RSE doses properly arranged in a linear fitting. The standard curve correctly met the 2 acceptance criteria prescribed by Ph. Eur., i.e., the regression

Tab. 1: Follow-up study on cell viability and responsiveness to RSE in cryopreserved 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 storage in liquid nitrogen.

	Cell viability (%)			RSE 0.2 EU/mL (IL-6 pg/mL)			RSE 0.4 EU/mL (IL-6 pg/mL)		
	6 months	12 months	18 months	6 months	12 months	18 months	6 months	12 months	18 months
PBMC_Donor 1	89.0	93.0	93.0	566	480	400	1200	1324	896
PBMC_Donor 2	91.2	95.5	95.5	500	455	475	1000	1000	1000
PBMC_Donor 3	95.0	92.0	91.0	410	370	430	1200	813	1200
PBMC_Donor 4	91.0	93.7	90.0	502	500	394	1213	1000	924

Fig. 2: Construction of the endotoxin and non-endotoxin standard curve in accordance with Ph. Eur. requirements

Peripheral blood mononuclear cells (PBMC) were stimulated with four doses of RSE (from 0.05 to 0.4 EU/mL) (A), FSL-1 (from 0.009 to 0.078 ng/mL) or R848 (from 0.075 to 0.6 mg/mL) (B). IL-6 response, reported as optical density (OD) values, was measured by ELISA. The evaluation of regression of response and of non-significant deviation from linearity was performed using Combistats. One representative experiment out of five conducted experiments yielding similar results is shown.

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 μ g/mL). Recovery of RSE or R-848 was measured by IL-6 release in terms of equivalent of endotoxin unit per mL (eEU/mL) or equivalent of R848 μ g per mL (μ g/mL), respectively, by ELISA. The range of valid eEU/mL or μ g/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).

of response was statistically significant ($p < 0.01$), as required by both Methods A and B, and there was no significant deviation from linearity ($p > 0.05$) as required by Method A only.

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 $2 \times$ AS. 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 Ph. Eur. 2.6.30 (EDQM, 2017), vaccine dilutions 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, which, when used in the test for interfering factors, allowed RSE recovery at the same dilutions as the vaccine (Fig. 3B).

Method validation for non-endotoxin contaminants

The main reason for considering MAT a reliable *in vitro* replacement of RPT is its unique capacity 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 non-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 μ g/mL of R848, corresponding to $2 \times$ 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 detection of NEPs.

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.

Standard		Standard+V1		Standard+V2		Standard+V3		Standard+V4		Standard+V5	
pg/mL	OD	OD	Interference (%)	OD	Interference (%)	OD	Interference (%)	OD	Interference (%)	OD	Interference (%)
600	2.629	2.549	-3.4%	2.507	-4.6%	2.580	-1.86%	2.415	-8.1%	2.535	-3.5%
300	1.788	1.770	-1.0%	1.818	+1.6%	1.579	-11.6%	1.719	-3.8%	1.662	-7.0%
150	1.095	1.061	-3.1%	1.024	-6.4%	0.924	-15.6%	1.001	-8.5%	0.980	-10.5%
75	0.593	0.555	-6.4%	0.565	-4.7%	0.500	-15.6%	0.562	-5.2%	0.526	-11.2%
37.5	0.294	0.240	-18.3%	0.291	-1.0%	0.244	-17.0%	0.276	-6.1%	0.265	-9.8%
18.8	0.140	0.131	-6.4%	0.130	-7.1%	0.111	-20.0%	0.116	-17.1%	0.120	-14.2%
9.38	0.076	0.073	-3.9%	0.062	-18.4%	0.068	-10.5%	0.068	-10.5%	0.066	-13.1%

Interference of TBEV vaccine in the detection system

Vaccine interference with the detection system for the chosen read-out also must be evaluated. To this aim, the 5 highest vaccine doses were added to the IL-6 standard curve. According to Ph. Eur. recommendations, all the detected OD variations due to vaccine addition at all the analyzed concentrations fell within $\pm 20\%$ of the OD detected with the recombinant protein alone (Tab. 2), indicating that the vaccine does not interfere with the ELISA detection system.

3.4 Determination of LOD, AS, CLC and MVD

To define the proper conditions for the TBEV vaccine-specific MAT optimization, we determined the LOD as well as the 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 RSE (Tab. 3). Similarly, very low amounts of both R848 and FSL-1 can be detected in our experimental setting as shown by LOD and AS values in Table 3.

By considering the parameters for parenteral adult and pediatric administration (see Section 2), the MVD for Encepur vaccine in the MAT assay was defined to be 1:2700 (Scheme 1¹). 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 (Tab. 4A,B and Fig. S1¹), a potential alternative for calculating the MVD may be to replace LOD with AS. By using AS, the proposed new MVD for the TBEV vaccine is 1:1000 (Scheme 1¹).

3.5 Application of Method A

Once all the MAT conditions were established, the vaccine was tested using Method A. This 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

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 (AS) of the linear part of the standard curve.

TLR agonist	LOD	AS
RSE (EU/mL)	0.040	0.100
R-848 (mg/mL)	0.050	0.150
FSL-1 (ng/mL)	0.003	0.009

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 RSE (corresponding to the middle dose of the endotoxin standard curve) were included (Scheme 2¹). Two different analysts performed the assay in a single analytical session and eEU/mL were determined for each vaccine dose, alone or combined with RSE, by using the linear part of the 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 amount of pyrogens detected in the vaccine resulted to be below the LOD, thus confirming the low level 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 found by both analysts, and thus the test was 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.

Tab. 4: Variance estimate and relative standard deviation for calculation of the precision of the method

(A) Repeatability of the method was evaluated based on the optical density (OD) values obtained from five different sessions, each performed by two analysts (total of 10) using PBMC from the same donor. The study was conducted on three RSE doses, namely 0.1, 0.2 and 0.4 EU/mL, corresponding to the assay sensitivity (AS), 2 x AS and 4 x AS, respectively. (B) Intermediate precision of the method was calculated based on the OD values obtained from a total of 20 different sessions performed by two analysts using PBMC from 8 different donors. The study was conducted on three RSE doses, namely 0.1, 0.2 and 0.4 EU/mL, corresponding to the assay sensitivity (AS), 2 x AS and 4 x AS respectively. SD, standard deviation; RSD, relative standard deviation

A

Component	0.1 EU/mL (AS)				0.2 EU/mL				0.4 EU/mL			
	Estimate (OD ²)	% of total	SD (OD)	RSD (%)	Estimate (OD ²)	% of total	SD (OD)	RSD (%)	Estimate (OD ²)	% of total	SD (OD)	RSD (%)
Day	0.117	79	0.342	/	0.045	72	0.212	/	0.002	11	0.039	/
Analyst	0.000	0	0.000	/	0.002	3	0.045	/	0.0002	1	0.014	/
Repeatability	0.030	21	0.174	19.8	0.016	25	0.126	6.1	0.012	88	0.111	4.3
Total	0.147	100	0.384	43.6	0.063	100	0.251	12.0	0.014	100	0.118	4.6

B

Component	0.1 EU/mL (AS)				0.2 EU/mL				0.4 EU/mL			
	Estimate	% of total	SD (OD)	RSD (%)	Estimate (OD ²)	% of total	SD (OD)	RSD (%)	Estimate (OD ²)	% of total	SD (OD)	RSD (%)
Analyst	0.000	0	0.000	/	0.000	0	0.000	/	0.000	0	0.000	/
PBMC donor	0.035	28	0.187	/	0.103	68	0.322	/	0.033	46	0.181	/
Assay	0.090	72	0.300	/	0.048	32	0.220	/	0.038	54	0.195	/
Intermediate precision	0.125	100	0.354	41.4	0.152	100	0.390	20.3	0.071	100	0.266	11.0

3.6 Application of Method B

Method B was 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 quadruplicates of 3 vaccine doses (1:100, 1:200 and 1:400) alone or spiked with 0.1 EU/mL of RSE (2 × LOD) and a 5-point dose-response curve of RSE, ranging from 0.025 to 0.4 EU/mL, was designed and is shown in Scheme 3¹. Even when the vaccine pyrogen level resulted to be below the LOD, no recovery of RSE was obtained when a spike of 2 × LOD was used for preparing endotoxin-contaminated samples, as indicated in Ph. Eur., and thus the test was not valid (Fig. 5A). However, since AS is the first value of the RSE curve allowing reliable evaluation, 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 × 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 to three different Encepur lots (data not shown).

3.7 Precision study

Repeatability of the method was 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 on 5 different days in the same laboratory and on cell samples from the same PBMC 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 IP (total variability) of the method was calculated based on 20 sessions, with PBMC samples from 8 different donors performed by 2 different analysts (Tab. 4B). It was evaluated by considering *Analyst*, *PBMC Donor* and *Assay* (i.e., analytical variability that accounts for *Within* and *Between assay* components) as random factors.

RSE dose 0.1 EU/mL, corresponding to the AS, slightly exceeded the predefined acceptance criteria (i.e., 20% for repeatability and/or 35% for IP) only for the IP (Fig. S2¹). This is conceivable since AS was established as one of the lower or the low-

Fig. 4: Application of quantitative Method A calculation to Encepur-optimized MAT assay

Peripheral blood mononuclear cells (PBMC) were stimulated with RSE ($R_1 = 0.05$, $R_2 = 0.1$, $R_3 = 0.2$ and $R_4 = 0.4$ EU/mL) or Encepur vaccine (1:100, 1:200 and 1:400) according to the plate layout presented in Scheme 2¹, and optical density (OD) values were measured by IL-6 ELISA. Statistical analysis was conducted 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 show OD derived from PBMC treated with vaccine spiked-in with 0.2 EU/mL of RSE. Numbers close to triangles indicate the percentage of RSE recovery. One representative experiment out of three experiments yielding similar results is shown.

est endotoxin dose where the linear fitting of the curve starts. Similar to the repeatability, also the IP of the assay proportionally increased with the increment of the RSE doses, as evident from the total RSD equal to 41.4, 20.3 and 11.0%, respectively.

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 IP), the contribution of the donors resulted to be the largest portion (equal to 68%; Tab. 4B) of the total variability, while contribu-

tion of the analysts resulted to be $\leq 3\%$, 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 the precision study indicated that the assay is very precise (RSD = 11%; Tab. 4B). Interestingly, the precision of the method increases linearly with the RSE dose as evident from the decrease of the RSD (Tab. 4A,B and Fig. S1¹). Overall, this method showed satisfactory precision.

Fig. 5: Application of semi-quantitative Method B calculation to Encepur-optimized MAT assay

Peripheral blood mononuclear cells (PBMC) were stimulated with RSE ($R_1 = 0.025$, $R_2 = 0.05$, $R_3 = 0.1$, $R_4 = 0.2$ and $R_5 = 0.4$ EU/mL) or Encepur vaccine (1:100, 1:200 and 1:400) according to the plate layout presented in Scheme 3¹, and optical density (OD) values were measured by IL-6 ELISA. Results obtained from an experimental session performed by two different analysts on PBMC of the same donor are shown. Squares indicate 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 RSE. Numbers close to triangles indicate the percentage of RSE recovery. The 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. One representative experiment out of three experiments yielding similar results is shown.

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

The idea of the MAT, previously called *in vitro* pyrogen test (IPT), was born more than two decades ago when the basic mechanism of fever induction had been revealed and cytokine detection by ELISA became a common tool in laboratories (Hartung and Wendel, 1995; Hartung et al., 2001; Gaines Das et al., 2004; Dinarello, 2004; Schindler et al., 2009).

According to European legislation (EU Directive 2010/63/EU), animal tests are to be questioned. To this aim, the EU national ministries and 3R grants made several financial contributions, which resulted in the validation of the MAT in 2005 by ECVAM (European Center for the Validation of Alternative Methods) (Hoffmann et

al., 2005) and implementation of chapter 2.6.30 “Monocyte Activation Tests” into the Ph. Eur. in 2010.

Nowadays, regarding vaccines, the 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 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 of 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 with respect to modified LPS (Gerke et al., 2015).

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 in-

trinsic 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 Ph. Eur. Method A and then Method B. After 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, as recommended by Ph. Eur. to ensure the validity of the test (EDQM, 2017), and then the final assay.

Indeed, given the expression of the wide receptor repertoire for microbial products, PBMC constitute a useful cell platform to reveal the presence of unknown pyrogens that may derive from the growth of contaminating microbes or as carry-over of an unsuccessful purification step during the manufacturing process, as 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 β and TNF- α , is fully released in culture medium (Hasiwa et al., 2013), thus providing a robust measure of pyrogen content.

In addition to both endotoxin and NEP detection, the possible interference of the vaccine with the technique clearly demonstrated that 1:100, corresponding to 0.03 $\mu\text{g/mL}$ of the product, is the first suitable vaccine dilution to be included in the final plate layout. Of note, the interference observed with vaccine doses higher than 0.03 $\mu\text{g/mL}$ 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. Chapter 2.6.30 in Ph. Eur. was written 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: According to 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. Replacing the LOD, which is variable from assay to assay, with AS, confirmed in every valid experiment, is a 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, m^2 or per eye (intravitreal application)) is needed, whose value is defined in exactly the same way in chapter

5.1.10 (Guidelines for using the test for bacterial endotoxins) and in the revised chapter 2.6.30 (Monocyte-activation test, EDQM, 2017). Nevertheless, for products like vaccines that are frequently administered intramuscularly in low volume, no K-value is specified in chapter 2.6.30. To overcome the issue of the 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 per kilogram of 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 is a critical assay validity criterion, especially in the presence of an NEP or an intrinsic pro-inflammatory product and, therefore, this requirement is very unlikely to be consistently fulfilled.

Indeed, even though 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 non-valid test result, notwithstanding the test sample data accomplished the product-specific limit of pyrogen contents (CLC) and the recovery of endotoxin.

In conclusion, our data provide evidence that the pyrogen level of the vaccine Encepur can be established by MAT with a satisfactory precision as evaluated by repeatability and IP of the method. However, it is necessary to re-evaluate the restriction of curve linearity with regard to the dilution range. This aspect will not negatively affect the already validated assays showing robust linearity. In addition, if AS is used instead of LOD, Methods A and B would converge into a semi-quantitative or limit test. This could be established for all products that are not intrinsically pyrogenic, need to be tested in a range of dilutions that includes MVD, 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 not intrinsically pyrogenic biologicals using meta-studies and surveys.

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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.

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