Vaccine 37 (2019) 3747–3753

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development and validation of a monocyte activation test for the control/safety testing of an OMV-based meningococcal B vaccine

Caroline Vipond [a,*], Janet Sutherland [b], Karin Nordgren [b], George Kemp [a], Alan Heath [c], Rory Care [a], Lucy Studholme [b]

[a] Department of Bacteriology, The National Institute for Biological Standards and Control, Blanche Lane, Potters Bar EN6 3QG, UK
[b] Department of Biotherapeutics, The National Institute for Biological Standards and Control, Blanche Lane, Potters Bar EN6 3QG, UK
[c] Department of Biostatistics, The National Institute for Biological Standards and Control, Blanche Lane, Potters Bar EN6 3QG, UK

ARTICLE INFO

Article history:
Received 21 February 2018
Received in revised form 15 June 2018
Accepted 17 June 2018
Available online 10 December 2018

Keywords:
Bexsero
Outer membrane vesicles
Monocyte activation test
Pyrogenicity

ABSTRACT

It is imperative to ensure biological products are free of contaminating pyrogenic material prior to administration to patients. Historically the rabbit pyrogen test (RPT) was used to screen for such contamination in medicines for intravenous delivery. This test was adapted for use to screen vaccines. However, some, including meningococcal vaccines containing outer membrane vesicles, are intrinsically pyrogenic. Indeed, this is the case for Bexsero which contains relatively high levels of endotoxin and other potential pyrogens such as lipoproteins and porins. The RPT proved a difficult method for measuring the pyrogenic content of Bexsero and differences between laboratories in different countries made repeat testing at the control laboratories problematic resulting in batches being wrongly identified as unsafe. At NIBSC a monocyte activation test (MAT) was adapted and validated as an alternative. This required setting of a specification in-house and deciding on a decisional procedure using multiple donors, allowing batches equally pyrogenic or less, than those batches shown to be safe in a clinical trial, to be certified as safe. The resulting format was a reference comparison method with an upper limit of 1.8 relative pyrogen units (RPU). The batch passed if an initial four donors had a response equal to or less than 1.8 RPU, if one donor is above this limit the batch was tested in a further four donors and seven of the eight must be equal to or below 1.8 RPU. If two donors have a response greater than 1.8 the batch failed.

1. Introduction

The control of contamination in biological preparations intended for the parenteral administration to humans is crucial given the adverse effects which can be caused if unintended pyrogens are present [1,2]. These can include fever, multiple organ failure and shock, potentially leading to death in severe cases. The rabbit pyrogen test (RPT) was originally developed to confirm the absence of pyrogens in large volume parenteral preparations. In accordance with the Pharmacopoeias, the test involves the intravenous infusion (IV) of the test drug into rabbits; the product passes the pyrogen test if the temperature increase of the rabbits is within a defined specification. More recently, this test has been applied to vaccines but its use for this purpose is limited [3]. The route of administration of vaccines to rabbits in the RPT (IV) and to human recipients (intramuscularly; IM, subcutaneously; SC, intradermally; ID orally or nasally) is inconsistent and therefore the results of the RPT may not reflect how a vaccine is tolerated in a human patient. The RPT has been used historically to measure the pyrogenicity of meningococcal outer-membrane vesicle (OMV) based vaccines [4–6] alongside the bacterial endotoxin test based on the clotting reaction of the hemolymph of the horseshoe crab [7]. The study by Rosenqvist et al. compared free endotoxin with OMV bound endotoxin in the presence of aluminium adjuvants demonstrated a difference in the RPT, the BET and in humans with free endotoxin giving the greatest response and OMV bound endotoxin adsorbed to adjuvant giving the least reaction [4]. The principles of this reduction of pyrogenic activity using adjuvant remain and meningococcal vaccines containing OMVs are adjuvanted partially to temper the reactivity of the endotoxin. Whilst the study, undertaken over two decades ago, was crucial for OMV based vaccines to be approved for use in humans, both tests are flawed; the RPT as it was designed solely to detect contamination and the BET as it detects only endotoxin. To permit the use of the RPT for an inherently pyrogenic vaccine, it must be diluted to a previously established non-pyrogenic dose determined from a clinically safe batch and used to identify any batches which

* Corresponding author.
E-mail address: caroline.vipond@nibsc.org (C. Vipond).

https://doi.org/10.1016/j.vaccine.2018.06.038
0264-410X/

are pyrogenic at the specified dilution. The test then becomes a consistency test since the administered doses are not comparable between the *in vivo* model and human patient. The principle of measuring consistency to ensure batches of vaccine are comparable to those shown to be safe in clinical trials is acceptable, as long as the attributes measured and the methods used are suitable. In the case of the RPT the inherent variability makes it difficult to apply as a consistency test. Indeed this has been the case at the National Institute for Biological Standards and Control (NIBSC) for a meningococcal (OMV)-based vaccine, Bexsero. To date, the OMV components in vaccines for human use are produced by detergent extraction, a consequence of which is the carryover of periplasmic and cytoplasmic components [8–13]. The resultant OMVs contain endotoxin (LPS) and non-endotoxin pyrogens (such as porins, muramylpeptide, bacterial DNA and peptidoglycan) either as intrinsic components in the outer membrane of gram negative bacteria, or as by-product of production and are inherently pyrogenic [14–16]. For many products, the BET is a suitable replacement for the RPT; however, since the BET does not detect non-endotoxin pyrogens, its sole use as a RPT replacement for testing Bexsero is inappropriate.

Given the limitations of the RPT and the BET, we were presented with the challenge of validating an alternative test for the control of pyrogenicity associated with Bexsero. Monocytic cells are mediators of the innate immune system and can be used to detect pyrogenic contaminants in medicines [17]. They express pattern recognition receptors (PRRs), including toll-like receptors (TLRs) which bind to specific pathogen-associated molecular patterns (PAMPS), resulting in monocyte activation and release of endogenous pyrogens (e.g. TNFα, IL-1β, IL-6, IL-8) which are mediators of the inflammatory response, often resulting in fever [18]. Since 1984, scientists have focused on the development and validation of *in vitro* pyrogen tests based on this premise [1,16,19–23] and in 2010, the Monocyte Activation Test (MAT) was included in the European Pharmacopoeia (Ph. Eur.; Chapter 2.6.30). In contrast to the RPT, the MAT can be applied as a fully quantitative test without the use of animals, making it more appropriate for vaccines which are inherently pyrogenic and is physiologically relevant since it uses human cells. Here we report the development and validation of the MAT as a replacement for the RPT, for the consistency/safety testing of Bexsero described above. Changing from the RPT to the MAT was also in line with the 3Rs principle [24] and EU Directive 2010/63/EU [25] concerning the protection of animals.

2. Materials and methods

2.1. Materials

2.1.1. Consumables and reagents

All consumables and reagents were purchased as sterile and pyrogen-free, unless used for IL-6 ELISA. Reagents for culture medium were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Gibco, Life-Technologies (Carlsbad, CA, USA) unless otherwise stated. All reagents were reconstituted and stored in accordance with the manufacturer's instructions. Lots of Bexsero were provided by the manufacturer; GSK vaccines (Siena, Italy), including a 200% sample in vaccine buffer. The 3rd WHO International Standard (IS) for endotoxin (10/178) and the 1st WHO IS for IL-6 (89/548) were obtained from NIBSC (South Mimms, Hertfordshire, UK). An in-house ELISA pair described previously was used for the IL-6 ELISA [1,19,27].

2.1.2. Blood donations

Human whole blood was donated by consenting employees of NIBSC performed under the local ethical committee approval and guidelines. Donation criteria and procedure was followed as described in Nordgren [28] with sufficient heparin (Sigma-Aldrich) to achieve 10 EU/ml. Leukoreduction system chambers (LRSCs) were received through NHS Blood and Transplant from John Radcliffe Hospital (Oxford, Oxfordshire, UK) on the day of apheresis donation and isolated PBMCs were cryopreserved within 10 h of donation as described previously [28].

2.2. Methods

2.2.1. Preparation of PBMCs

Isolation of PBMCs from whole blood was carried out as previously described with minor modifications [1]. Briefly, the PBMCs were purified by density gradient centrifugation using histopaque-1077 (Sigma-Aldrich), the PBMC layer isolated and washed three times with PBS (Ca^{2+} and Mg^{2+} free) followed by resuspension in culture medium (RPMI supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, MEM non-essential amino acids, 2 mM L-glutamine and 2% (v/v) human AB serum (Biosera, Nuaille, France)) to 1×10^6 cells/ml. PBMCs were counted using Trypan blue with a Countess™ automated cell counter (Invitrogen) or haemocytometer. If cryopreserved PBMCs isolated from LRSC were used, thawing was performed as described previously [28] and resuspended to the same cell number. 125 μl of cell suspension were added per well of 96-well round-bottomed polypropylene cell culture plates (Corning, Acton, MA, USA).

2.2.2. Preparation of test, reference and endotoxin solutions

Syringes of Bexsero and 200% sample were gently vortexed for 15 s and dispensed into borosilicate glass tubes (Charles River Laboratories, Wilmington, MA, United States). Endotoxin IS dilutions were prepared from a 2000 IU/ml stock and vortexed for 2 min. Samples were serially diluted in culture medium in borosilicate glass tubes with each dilution vortexed for 2 s prior to preparation of the subsequent dilution. 125 μl of the solutions were added in quadruplicate to the cell culture plates directly after preparation. A negative control (culture medium only added to cells) and positive control (Endotoxin IS at final concentration of 0.5 IU/ml) were included on each plate. The cultures were incubated for 18–21 h in a humidified 37 °C incubator, with 5% CO_2 (v/v). A placebo buffer containing sodium chloride, histidine, sucrose, water and aluminium hydroxide adjuvant, without recombinant proteins or OMVs was provided by GSK to test for any background reactivity not associated with the active antigens in Bexsero.

2.2.3. Determination of IL-6 in supernatants

Supernatants from the cell culture were collected and analysed for IL-6 content directly using a sandwich ELISA or stored at −30 °C until assayed. An in-house IL-6 antibody pair, mouse monoclonal capture antibody and sheep polyclonal detection antibody directly conjugated to HRP, were used to quantify IL-6 in cell culture supernatants (diluted 1/10 in culture medium) as described previously [27]. TMB was used as HRP substrate and the reaction stopped using 1 M H_2SO_4. The absorbance values were measured at 450 nm using 540 nm as a reference wavelength using SpectraMax 340PC microplate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve was generated in each assay using IL-6 IS diluted in culture medium from 62.5 to 4000 pg/mL.

2.2.4. Data analysis

Dixon's Test ($\alpha = 0.02$) was used to exclude outliers within the 4 replicates for each sample/reference dilution. To determine the relative pyrogen units, parallel-line analysis of the dose-response curves for the test and reference samples (on the same test plate) was carried-out using CombiStats™ (European Directorate for the Quality of Medicines, Council of Europe, version 5.0). Of five

dilution points, the 3 which generated the steepest slope were used for parallel-line analysis. The reference batch was assigned a value of one. Sample dilutions giving a mean OD value below the mean of the negative control tested were excluded from the analysis. Validity criteria of: regression; $p < 0.05$, non-linearity; $p > 0.01$, non-parallelism; $p > 0.01$ were applied relative to the reference batch.

3. Results & discussion

3.1. Setting a specification for the MAT

Ph. Eur. Monocyte Activation Test Chapter 2.6.30 methods A and B involve comparing responses to the test product with responses to an endotoxin standard to calculate an endotoxin equivalent value (EE per mg or ml) for the pyrogen(s) being measured. The test product passes if the EE value is below specification, which is the same value as the endotoxin limit concentration (ELC) described for the BET (Ph. Eur. Chapter 2.6.14) but is referred to as the contaminant limit concentration (CLC) in the MAT, since the nature of the pyrogen is usually unknown. Preliminary data showed that with the majority of PBMC donors, the observed dose-response curves for test batches of the OMV-based vaccine were not parallel to those of the 3rd WHO International Endotoxin Standard (Fig. 1). Consequently, an accurate assignment of a value in EE could not be calculated. The method developed at NIBSC for this vaccine was therefore based on the more appropriate Ph. Eur. MAT Method C: Reference Lot Comparison Test in which a well-characterised batch of the product is used instead of standard endotoxin as the reference. Whilst Ph. Eur. Method C at the time did not prescribe a specific method of analysis to calculate 'potency' relative to the reference batch, NIBSC developed an 'in house' method of analysis. For this, five 2-fold dilutions each of the test and reference batches are assayed on a test plate, of which three dilutions in the linear part of the dose-response curves are selected for a parallel line analysis using CombiStats to assign a relative pyrogen unit (RPU) to the test batch. This is shown in Fig. 2.

In order to set the pass/fail specification five batches which had been demonstrated to be safe in clinical trials, in infants, toddlers, children and adolescents were used. In addition GSK provided a batch of vaccine which had failed in the RPT three consecutive

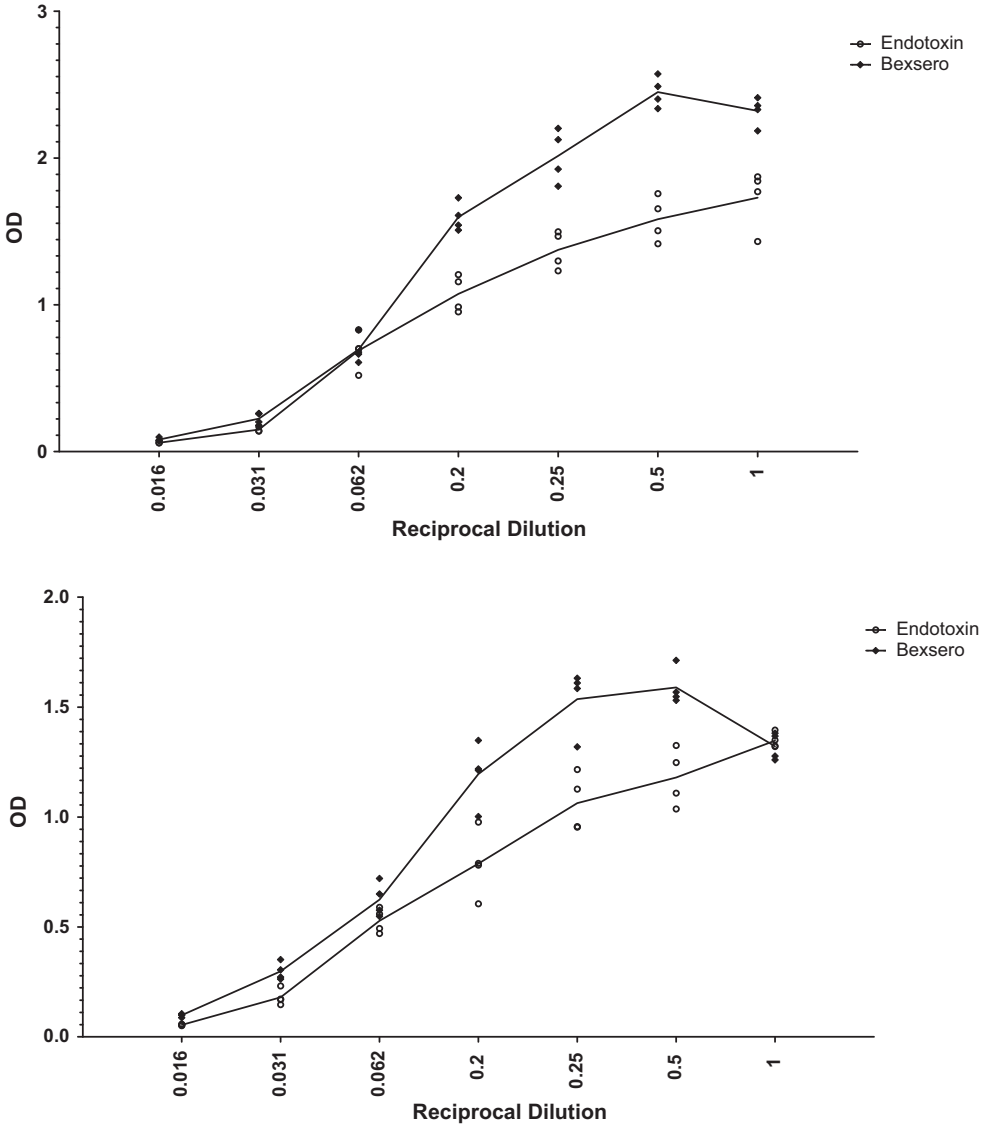

Fig. 1. Examples of the dose response curves from donor PBMC treated with either Bexsero or endotoxin. For the endotoxin curve the highest dilution is 6.4 IU/ml; for Bexsero the highest dilution is 1 in 100 of a standard human dose.

times and was considered to be more pyrogenic than other batches produced during the early stages of Bexsero production, for the remainder of this manuscript this will be referred to as the control high batch. The five clinical batches were initially tested with PBMCs from 4 donors, results (activity relative to the reference batch) are shown in Table 1.

To determine a more accurate estimate of both the mean RPU and spread of response in the donor population, two clinical batches (1 and 5) were selected, informed by results of the initial MAT in 4 donors and were tested in an additional 16 donors. All tests were valid at a 1% significance level for non-linearity and non-parallelism, and 5% significance value for regression with one exception. This was a test with a single donor of the control high batch of vaccine, where the dose-response lines were significantly non-parallel ($p < 0.01$). For information, the estimated potency from this invalid test is also included in Table 1. Batch 1 was selected as it showed greatest donor variability and the highest response in a single donor and batch 5 as it induced the highest geometric mean (Table 1). Distributions of natural log of the relative potencies across the 20 donors are shown in Fig. 3 for clinical batch 1 and clinical batch 5.

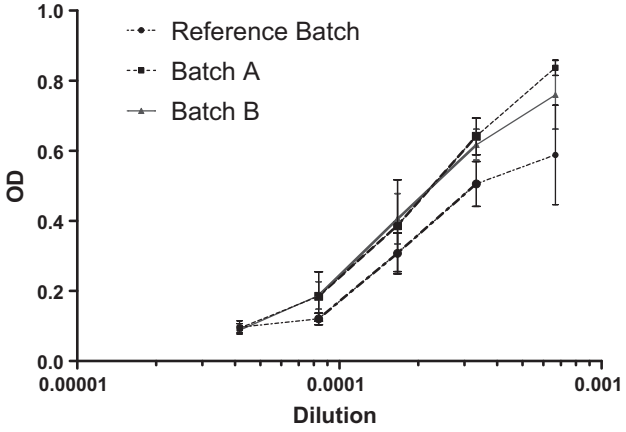

Fig. 2. Example of dose response curve from a Bexsero MAT showing reference and two test batches. The bold lines mark the three doses used for the parallel line analysis to assign test batches with a relative pyrogen response.

There is some evidence that these distributions have a positive skew (i.e. a few high values causing a lack of symmetry) but there are no significant departures from normality as determined with the Anderson-Darling test with a p-value of 0.215 and 0.18 respectively (where a p value of > 0.05 is deemed normal). Assuming a normal distribution of the estimated natural log potencies across donors, the inverse cumulative distribution function was used to calculate an upper value assuming 1 sided distribution to cover both 95% and 99% of the population. An estimate of the between donor SD was calculated as a pooled estimate from both distributions. The 95% and 99% upper values were 1.57 and 1.75 respectively. These predicted upper limits fit well with the observed maximum of 1.67, and the observed 95th percentile (19th highest out of 20) of 1.6. The MAT is a variable assay, the distribution of results reflects this and is a combination of assay variability and real differences between responses of individual donors. Clinical batch 5 gave the highest relative pyrogen response of all five clinical batches, and as this was tolerated in infants in the clinic it was deemed that any batch giving an RPU value equal to or less than the highest expected value seen in donors would be acceptable for use. Therefore a rounded limit of 1.80 was adopted as the maximum allowable RPU response for any individual donor for batches of Bexsero. Using these criteria all donors would have an acceptable RPU in response to clinical batch 5 (Table 1).

3.2. Decisional procedure

For a MAT procedure which involves the use of individual donations of blood/PBMC (as opposed to cells pooled from a number of donors), the Ph. Eur. states that the batch is tested using 4 different

Table 1
MAT data showing individual donor responses, expressed in relative pyrogen units, to the clinical trial batches tested in the MAT. *Result not conforming to statistical acceptance criteria detailed in Section 2.2.4.

	Clinical trial batches					Control high batch
	Clinical batch 2	Clinical batch 3	Clinical batch 4	Clinical batch 1	Clinical batch 5	
Donor 1	0.72	1.01	1.05	0.95	1.24	1.58
Donor 2	1.02	1.11	0.98	1	1.14	1.55
Donor 3	0.83	1.02	1.14	0.99	1.31	1.34
Donor 4	0.83	1.15	0.87	1.34	1.25	2.1
GM	**0.84**	**1.07**	**1.01**	**1.06**	**1.23**	**1.62**
%CV	**14.9**	**6.4**	**11.5**	**16.3**	**14.9**	**20.6**
Donor 5				1.22	1.05	1.6
Donor 6				1.29	1.13	1.72
Donor 7				1.47	1.6	1.05
Donor 8				1.22	1.34	2.74
Donor 9				1.19	1.3	2.01
Donor 10				1.04	1.14	1.8
Donor 11				1.16	1.1	2.37
Donor 12				0.74	1.22	1.86
Donor 13				1.07	1.37	1.72*
Donor 14				1.2	1.34	1.78
Donor 15				1.25	1.67	1.46
Donor 16				0.9	0.94	2.03
Donor 17				1.17	0.98	
Donor 18				0.83	1.11	
Donor 19				0.97	1.11	
Donor 20				1.06	1.2	
			GM	1.09	1.21	1.75
			%CV	16.6	15.1	23.8
			P SD		1.17	

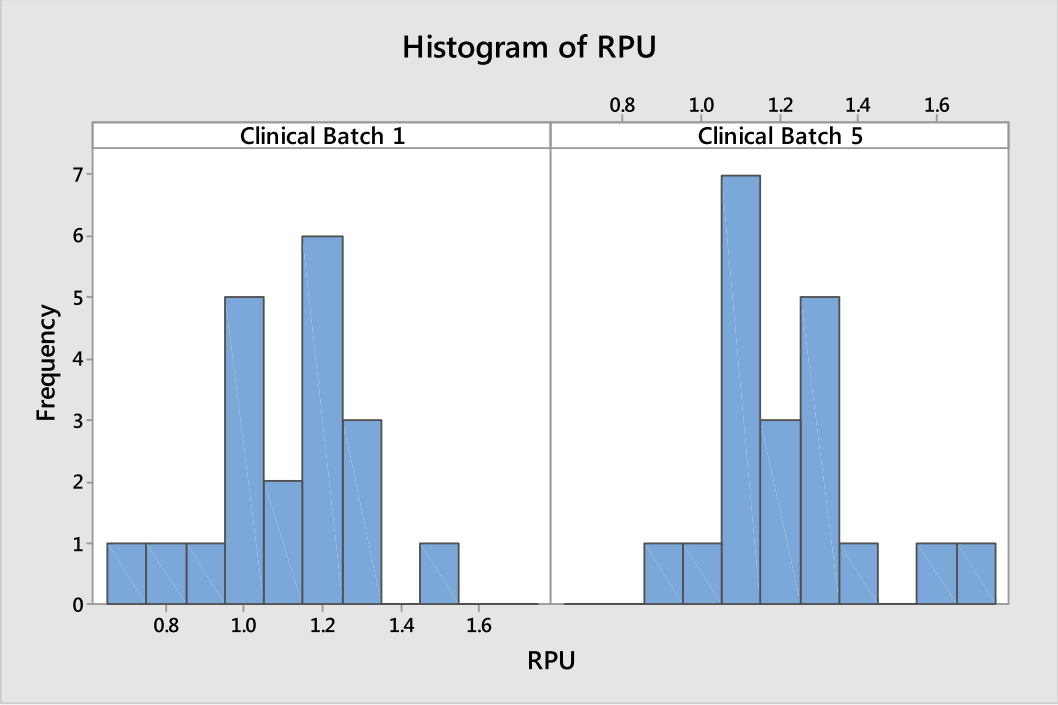

Fig. 3. Distribution of long transformed relative pyrogenicity units in 20 donors for clinical batches 1 and 2.

donors and if the results from all 4 donors comply with the specification, the batch passes. If the batch complies with 3 of the 4 donors, then the test is repeated with a further 4 different donors. Provided all 4 of these new donors comply with the test, then the batch passes (i.e. no more than 1 out of 8 unacceptable results). Any other outcomes result in a fail for the test batch. Therefore, a deviation from the normal batch composition only detected with 2 donors out of a group of 8 would result in a 'fail' even if results averaged across all donors were acceptable. NIBSC adopted this pass/fail decision procedure to encompass the potential differences in response to different pyrogenic contaminants across different donors.

Therefore, with the selected 99% upper limit (as described above), if all four donors give a result equal to or below 1.8 in the first assay, the batch passes. If one donor gives a result greater than 1.8, the batch is tested using a further four different donors and must give a result equal to or less than 1.8 with all four donors (i.e. with 7/8 donors for the entire test), for the batch to pass. Using an overall mean (of activity relative to the reference batch) across donors would still be valuable for measuring batch to batch consistency and monitoring any trends in product or assay performance.

3.3. Test validation

As a Ph. Eur. test, the MAT is considered to be validated and fit for purpose as a replacement for the rabbit pyrogens test. Here we address the product specific aspects of validation including test reproducibility (precision), linearity, range and specificity.

3.3.1. Reproducibility

Three batches of Bexsero were tested in at least two independent assays by two operators. The GCV of each of the batches was less than 20% (Table 2). The data was further analysed to evaluate the sources of variability associated with the assay (Table 3). The variability between the donors was the biggest contributor, accounting for 67% of the variation observed.

3.3.2. Linearity and range

The RPU values for three different concentrations of Bexsero ranging from 50% of a single human dose (SHD) to 200% of a SHD were determined in eight individual donors. As presented in Fig. 4, there is good agreement between the expected and observed values. As the concentration (% Bexsero) decreased, RPU values significantly regressed ($p < 0.001$, ANOVA regression analysis) and there was no evidence of significant non-linearity ($p > 0.05$, ANOVA test for non-linearity). These results were also confirmed when cells which had been cryopreserved cells were used in the assay.

Table 2
Summary of reproducibility data. GM: geometric mean, n: number of individual test results for the batch of vaccine, LCL/UCL: lower and upper confidence limit, GCV: geometric coefficient of variation. For each PBMC donation, all batches had an RPU $\leq$ 1.8 and met test validity criteria. The GCV for all three batches were below 30% (acceptance criteria).

	Batch 1	Batch 2	Batch 3
GM	1.09	1.00	1.12
(n)	(20)	(20)	(16)
95% LCL	1.01	0.94	1.05
95% UCL	1.17	1.06	1.20
GCV	17%	14%	14%

Table 3
Sources of variability associated with the MAT (calculated using SAS).

Variance component	Description	Estimate	%
Total	All	0.00456	100
Assay	Within laboratory	0.00071	16%
Error	Donor to donor	0.00306	67%

Note: the remaining 17% variability (estimate 0.00078) is accounted for by differences in the three batches tested. The inclusion of "batch" as a variance component allowed an overall estimate for "Assay" and "Error" to be calculated, as opposed to individual estimates for each batch.

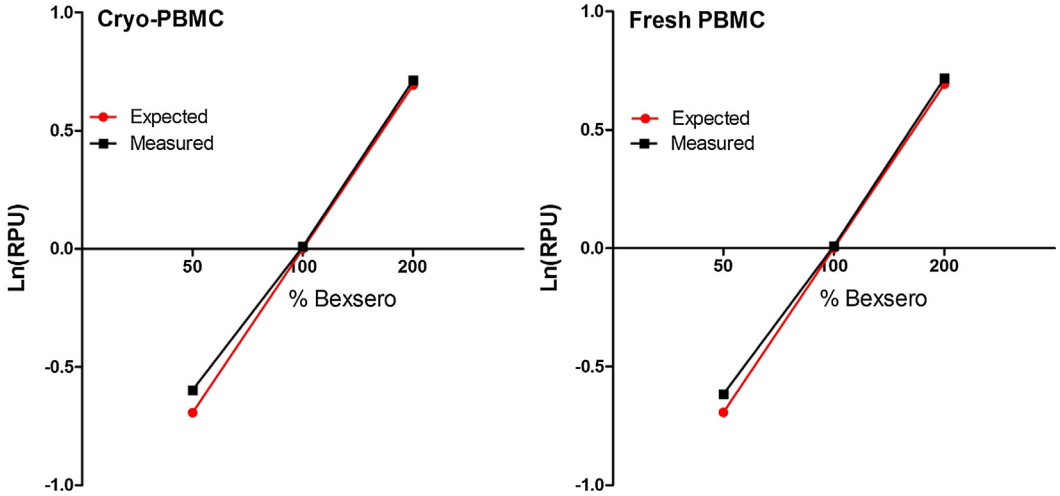

Fig. 4. Observed and expected RPU values for three different concentrations of Bexsero (%) tested in the MAT. Observed values are geometric mean RPU values of 8 PBMC donations.

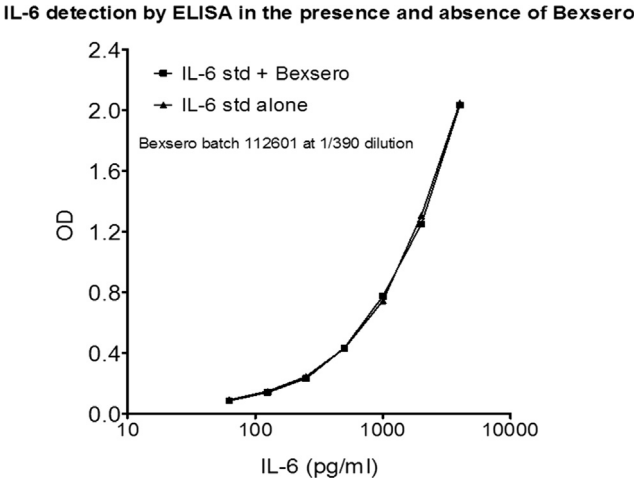

Fig. 5. Optical density (OD) values for IL-6 in ELISA method with or without Bexsero. Bexsero does not interfere with IL-6 detection.

3.3.3. *Specificity*

An IL-6 standard preparation (89/548) was tested in the IL-6 ELISA in the presence and absence of Bexsero and the curves differed by less than 20% (Ph. Eur. specification) indicating the vaccine does not interfere with the cytokine detection method (Fig. 5). In addition, the buffer used to formulate Bexsero but without OMV and recombinant protein was tested in a MAT and did not stimulate an IL-6 response in cells above that of the cells incubated with culture medium alone. Therefore, any response in the MAT cannot be attributed to the components of the formulation buffer alone.

4. Conclusions

The PBMC IL-6 MAT has been long established at NIBSC [20], chosen because: PBMCs were found to give better results with increased sensitivity as compared to whole blood [19,23]; an increase in the release of IL-6 correlates with endotoxin induced fever in rabbits [3], and it is secreted entirely into culture medium unlike IL-1β and TNFα where a proportion of which stays within the cells. Therefore, this format was used to develop the MAT for Bexsero [16]. However, work is ongoing to establish if this complex vaccine stimulates other toll-like receptors giving rise to other cytokines which would very likely play a role in the stimulation of the innate immune system as clinically observed for this vaccine. Since the initial validation of the method with freshly isolated PBMC, we have introduced the use of PBMCs isolated from leukoreduction system chambers, then cryopreserved. PBMCs prepared in this way have shown comparable sensitivity, linearity and range compared to fresh PBMCs as illustrated in Fig. 4, with improved accessibility to cells, ease of use and reproducibility.

Two approaches could have been applied when setting criteria for the MAT either setting of an upper limit, or with constraints set on variation. As Bexsero was a new product known to cause fever in infants [29,30] it was decided that the responses of each individual donor would be evaluated rather than using a mean result of all donor responses. In addition a consistency based approach, using constraints on variation, would require a large population of batches to be tested to enable setting of a specification which captures the variability of batches of Bexsero over a prolonged manufacturing period. Complications in the safety test approach used in this study, i.e. where an upper limit was set, were the limited availability of known unsafe batches to determine suitable discriminatory limits, which would distinguish a safe from an unsafe batch of vaccine. Setting the cut-off value of the RPU was a balance between identifying batches with elevated pyrogenicity and failing potentially safe batches by chance alone. Applying the specification to the clinical trial batch which gave the highest potency in the MAT, an estimated 99.4% of the donor population would have a potency estimate below the 1.8 cut-off. Consequently, it was predicted that only 0.08% of batches with a potency estimate equal to the batch would fail. Whilst there is no evidence that the control high batch, as defined by GSK, is clinically unsafe, the 1.8 cut-off maintains the power to discriminate this batch as more pyrogenic in 92.2% of tests. As more batches are released and used in a clinical situation, the amount of data available from known 'good' batches has increased, and the proposed limits and approach of setting the specification will be reviewed and updated. The cut-off value set for the test is stringent; however, prior to the introduction of the MAT, pyrogen testing at the OMCLs was in the form of the RPT and a high number of batches were failing. The MAT is a more appropriate test and following implementation batch release of batches of Bexsero was faster, with significantly fewer false positive batches whilst also providing a more meaningful result. In addition, the replacement of the RPT and BET with the MAT is in line with the 3Rs principles, preventing the need for using either rabbits or horseshoe crabs for routine testing of

Bexsero. In future, we hope that testing of other vaccines will also consider this test as an alternative.

In conclusion, we have successfully validated the MAT for the testing of Bexsero; we have justified the pass/fail criteria for testing, in accordance with other Ph Eur methods, and the assignment of a suitable cut-off. In the future, it is important to assess the robustness of the assay as described in the Ph Eur in all laboratories and to review the appropriateness of both the pass/fail criteria and specification once a large dataset of results have been generated. We highlight the importance of carefully considering the properties of a biological medicine, and how it will be used, when defining quality control testing to avoid the use of methods chosen because they are written into the Pharmacopeia without assessing if they are also scientifically the most suitable.

Note added to proof

Bexsero has been used routinely in the UK since September 2015 with a very good safety profile as assessed by Vigilance and Risk Management of Medicines, MHRA [26]. With this known safety profile and experience of hundreds of batches of vaccine the pass/fail specification has been revised and uses a mean value of four or eight donors with a revised upper limit.

Acknowledgements

We would like to thank colleagues Svein Rune Andersen, Therese Solstad Saunders, Hilde Ohldieck Jarstadmarken and Anne Dybwad from the Norwegian Medicines Authority for support and assistance in the development of the MAT for Bexsero. Also, colleagues Barbara Capecchi, Leonardo Gherardini and Davide Serruto, GSK vaccines for their critical review of the paper and for support and assistance in developing the MAT.

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