Review Article

Limitations of the Rabbit Pyrogen Test for Assessing Meningococcal OMV Based Vaccines

Caroline Vipond[1], *Lucy Findlay*[2], *Ian Feavers*[1] *and Rory Care*[1]

Department of Bacteriology[1], Department of Biotherapeutics[2], National Institute of Biological Standards and Control, South Mimms, Potter Bar, UK

Summary

The rabbit pyrogen test was developed in the early 1900's to detect contaminating pyrogens in parenteral medicines. Since its conception alternative methods with improved sensitivity, relevancy and which are ethically more acceptable have been developed. However, the test is still a current pharmacopeial method and is used to evaluate the pyrogen content of some vaccines. In this article the limitations and pitfalls of using the test to measure pyrogenicity of vaccines containing outer membrane vesicles are described. The method is unsuitable as a safety test for these products due to the high levels of endotoxin present in the vaccine which generate a pyrogenic response in rabbits when delivered intravenously without dilution. Its use as a consistency test is also ambiguous as the test gives a qualitative rather than quantitative response, and the rabbit models are highly variable. In addition there is evidence that measuring the temperature rise of the animals over three hours does not capture the maximum fever response. Finally the article considers the use of alternative methods and the validity of animal models when applying a consistency based approach for assessing the quality of licensed products.

Keywords: outer membrane vesicles (OMV), meningococcal vaccine, rabbit pyrogen test (RPT), monocyte activation test (MAT)

1 Introduction

The rabbit pyrogen test is used, both by vaccine manufacturers and national control laboratories, to assess a number of vaccines for their pyrogen content, including the multivalent diphtheria, tetanus and Hepatitis B vaccine, Hepatitis B, rabies, tick borne encephalitis, and those based on meningococcal outer membrane vesicles (OMVs). The test was originally designed for large volume, intravenously administered products, so the suitability of the RPT for vaccines administered intramuscularly or subcutaneously is questionable. On the one hand, many vaccines are pyrogen free and the use of the RPT does not present a problem, although its relevance to the clinical situation remains unclear. But in the case of meningococcal OMV based vaccines, which intrinsically contain relatively high concentrations of pyrogenic material, and vaccines containing adjuvants that are known irritants, using the RPT is more problematic. This article considers why the test is used from a historical perspective, the different reasons why it is not suitable, and presents previously unpublished data which illustrate one of the issues and suggest an alternative cell based test to replace the RPT in the future. The implementation of the alternative test to replace the RPT concurs with the principles of the three R's (replacement, reduction and refinement) tenet.

2 History of the RPT

The rabbit pyrogen test (RPT) is a qualitative method used to detect the presence of contaminating (fever-causing) pyrogens in parenteral preparations by measuring temperature changes in rabbits following administration of a test sample. Its first recorded use was in 1912 by Hort and Penfold whilst investigating the origin of pyrogens that were causing fever in people being treated with "injectables" (Hort and Penfold, 1912). In the 1920's Seibert, Bourn and Mendel confirmed the contaminating pyrogens to be of bacterial origin and made further refinements to the test (Seibert, 1925). The high demand for intravenous solutions during the second world war led to an increase in the use of the test, which was included in the

Received September 29, 2015;
Accepted November 11, 2015;
Epub December 2, 2015;
http://dx.doi.org/10.14573/altex.1509291

US Pharmacopeia in 1942 (Roberts, 2007). It has been refined over the years and the pharmacopeial methods were updated to include screening of rabbits prior to use (Martin and Marcus, 1964) to reduce the number of false positives. However, the RPT has been critically scrutinized for its scientific and ethical shortcomings, and in many cases has been replaced with the bacterial endotoxins test (BET). However, as a long established pharmacopeial method, the RPT is considered an "industry standard," in particular for detecting non-endotoxin pyrogens (NEPS) in parenteral medicines.

3 Testing vaccines using the RPT

The purpose of an animal model often is to mimic the human response to disease and medicines. Rabbits were chosen for use in the pyrogen test because their sensitivity to endotoxin is similar to that of humans (Greisman and Hornick, 1969). The intravenous (IV) route of administration prescribed in the pharmacopeias for the RPT mimics the way that large volume parenteral drugs, for which the test was first designed, are administered. However, there are fundamental differences between the administration of IV drugs and vaccines to patients, stemming from the distinct pharmacodynamics of these products. Intravenous delivery ensures fast delivery of medication directly into the circulation and is often used in life threatening situations. Conversely, vaccines are administered subcutaneously (SC) or intramuscularly (IM) where they interact with cells of the immune system but absorption to the bloodstream is much slower. Moreover, sensitivity to pyrogens is much greater when delivered IV, an essential consideration when modelling a response to endotoxin.

Large volume parenterals must not contain greater than 5 IU endotoxin per kg body mass (World Health Organization, 2013), which equates to the sensitivity of the RPT, which was designed to be highly sensitive but not quantitative. For the BET, vaccines have a product specific limit of maximum allowable endotoxin content, which can be thousands of times greater than that for intravenously administered products, and is defined for quality control purposes. However, as the RPT is a qualitative assay, it cannot provide a definitive value (Hartung et al., 2001). The use of the RPT for evaluating vaccines known to contain relatively high levels of endotoxin, such as killed whole cells or outer membrane vesicle (OMV) based vaccines, is therefore problematic because of its sensitivity and the different route of administration.

Vaccines are different from most other medicine, being used prophylactically and in pediatric programs are given to healthy infants. Safety tests are of the utmost importance for vaccines, as administration of a contaminated product containing high concentrations of pyrogenic material could have devastating consequences. Pyrogenic material could originate from growth of contaminating bacteria or carry over from manufacturing processes in which purification is not completely successful. Bacterial endotoxin is of major concern to the pharmaceutical industry due to its high pyrogenicity, ubiquitous sources and stability (Hoffmann et al., 2005b). The BET, based on the clotting reaction of hemolymph from the horseshoe crab (Bang, 1956), was introduced into the US Pharmacopeia in 1980 and replaced the RPT for many products (Williams, 2007). The advantages of the BET include: quantitative or semi-quantitative measurement of endotoxin, high sensitivity, relative ease and speed of running the assay and, to some degree, the reduction in use of animals, although the critical LAL reagent is still of animal origin. In the case of vaccines, where an allowable limit of endotoxin is used as a license specification, the use of a quantitative assay offers a considerable advantage and also allows the consistency of subsequent batches of vaccine to be monitored. For the majority of vaccines this is now the accepted test method for assessing vaccine pyrogenicity. The principal disadvantage of the BET is that it does not detect non-endotoxin pyrogens.

Plain polysaccharide vaccines, developed in the 1960's by Gotschlich and co-workers, were the first successful meningococcal vaccines (Goldschneider et al., 1969a,b). The active component is the polysaccharide and ideally these vaccines should not contain any contaminating endotoxin. However, the purification processes used in the early days of manufacture did not remove all the residual contaminating endotoxin (Kuronen et al., 1977). An example of endotoxin contamination was documented in Finland, where a plain polysaccharide vaccine was used to control an epidemic of group A meningococcal disease. A number of batches of the group A polysaccharide vaccine caused febrile reactions in infants, which were attributed to the endotoxin content (Kuronen et al., 1977). The recommendation from this study was to implement the RPT. However, for the next generation polysaccharide vaccines conjugated to a carrier protein, the RPT was replaced with the BET assay, with the assumption that these vaccines were free from NEPs. To date there have been no adverse events reported to suggest contamination with NEPS.

4 Use of the RPT in meningococcal OMV based products

In the specific case of meningococcal OMV based vaccines the RPT has been used historically to measure the pyrogenic dose of the vaccine, i.e., the vaccine is diluted until a pyrogenic response is no longer seen in rabbits (Fisseha et al., 2005; Frasch and Peppler, 1982; Tsai et al., 1989; Zollinger et al., 1978). Meningococcal OMVs are composed of outer membrane proteins, lipids and lipopolysaccharide (LPS) or endotoxin. Endotoxin is, therefore, intrinsically part of an OMV vaccine. When the first developmental OMV vaccines emerged, the BET was a new test just being introduced to the pharmacopeias, making the RPT the pyrogen test of choice. Once the BET became a recognized pharmacopeial method it was used alongside the RPT in the quality control of these products. In the case of MenBvac, an OMV vaccine used to control an outbreak of disease in Norway in the late 1980s, the specification required that the product was non-pyrogenic at a dose of 1µg protein per kg of rabbit (equivalent to 1/50 standard human dose per kg of rabbit) (Frasch et al., 2001; Fredriksen et al., 1991). This was consistent with guidelines written for plain polysaccharide meningococcal vaccines, where the test product must be non-pyrogenic

Tab. 1: Overview of three most commonly used pharmacopeial rabbit pyrogen tests

Pyrogen test	Cumulative number of rabbits	Mean maximum response
European Pharmacopoeia	3, 6, 9, 12	0.41°C
Japanese Pharmacopoeia	3, 2	0.60°C
United States Pharmacopeia	3, 5	0.55°C

in the RPT when a defined amount of polysaccharide per kg of rabbit is administered (European Pharmacopoeia, 2005; World Health Organization, 1976).

To date OMV based vaccines are tested using both methods, the BET to measure the level of endotoxin present and the RPT to detect endotoxin, NEPs and any synergism between the two that may be present. The OMVs included in vaccines for human use are produced by detergent extraction (Bjune et al., 1991; de Moraes et al., 1992; Oster et al., 2005; Sierra et al., 1991), which removes lipoproteins and some LPS. However, a consequence of the detergent extraction is the carryover of cytoplasmic and periplasmic components (Ferrari et al., 2006; Vipond et al., 2006). Other known NEPs include porins, peptidoglycan, muramylpeptides and bacterial DNA (Hasiwa et al., 2013), all of which could conceivably be contained within OMV preparations either as genuine membrane components or as contaminants from the production process. In an attempt to detect the presence of any NEPs present in the vaccine that would not be detected in the BET, a modified RPT is used during pre-clinical testing, in quality control testing performed on clinical trial batches, and finally in routine control testing following licensure.

5 Consistency or safety test

If the RPT is to be used to measure the pyrogen content of a vaccine containing OMVs, the challenge dose (µg/kg) used should be the maximum non-pyrogenic dose obtained for lots found to be safe (non-reactogenic or acceptably reactogenic) in clinical trials. The rationale for this consistency based approach is that the test should discriminate a batch which is more pyrogenic than those used in clinical trials. The dilution of the vaccine to the equivalent of 1 µg of total protein per kg of rabbit, as suggested by Frasch et al. (2001), was the dose used for the quality control testing of MenBvac. However, MeNZB, a vaccine used to control an epidemic in New Zealand in the 2000s derived from a different parent strain, was more pyrogenic and required a greater dilution with a challenge dose equivalent to 0.214 µg protein per kg, to give a negative response in the RPT (Medsafe, 2006).

The addition of the dilution step makes the link between the test result and the safety of the undiluted product when administered to a human more tenuous. The general safety test or abnormal toxicity test is traditionally used to measure vaccine safety. This test is documented in WHO guidelines as well as various pharmacopeias. In the European Pharmacopeia the test involves administration of 1 human dose but no more than 1ml of vaccine intra-peritoneally to five mice and two guinea pigs (European Pharmacopoeia, 2008). The animals are observed for 7 days for signs of ill health most frequently measured by death rate and abnormal body weight changes. A key difference between this test and the RPT is that a standard human dose is administered to the animals to maximize the chance of observing an adverse reaction due to the content of any toxic material. The same rationale is used when testing large volume parenterals (which are usually pyrogen free or contain low levels of pyrogenic material), where as much as possible is administered to the rabbit to maximize the chance of identifying any unwanted toxicity. In the case of the RPT for the intrinsically pyrogenic OMV based vaccine, the product is diluted at least 25 fold, which is not representative of the human dose. The consequences of diluting the product have been recognized both by manufacturers and national control laboratories and this test is considered to be a consistency rather than a safety test. However, consistency is difficult to measure using *in vivo* tests that are inherently variable. The design of the RPT varies between pharmocopeias and the algorithms by which a pass/fail decision is reached also differ. All were set up specifically to identify contamination of a product but also to allow for the occurrence of a false positive, i.e., one animal which shows an increased temperature by chance. The three most commonly used algorithms from the European Pharmacopoeia (EP), United States Pharmacopeia (USP) and Japanese Pharmacopoeia (JP) have been evaluated by Hoffmann et al. (2005a). The three methods are summarized in Table 1.

Regardless of the differences between the methods, all are designed to identify contamination in a qualitative rather than quantitative manner for products that are usually free from or contain low-levels of pyrogenic contamination. To achieve a test with the statistical power to resolve differences between two batches of a pyrogenic product, the test algorithms need to be reviewed. The current pharmacopeial methods do not have the statistical power required for a discriminatory test. To achieve this power the number of animals would need to increase considerably, which is practically and ethically not acceptable.

6 Alignment of models between laboratories

The pharmacopeias set out the methods for pyrogen testing, but the requirements for the rabbits are vague. The USP stipulates healthy mature rabbits individually housed, whilst the EP requires healthy adults of either sex weighing no less than 1.5 kg.

However, the sensitivity of rabbits to pyrogens differs depending on the strain, the sex, and environmental conditions such as housing and husbandry (Hull et al., 1993; van Dijck and van de Voorde, 1977) and even when the test is carried out, in terms of both time of day and year (Bellentani, 1982). It is therefore possible for two laboratories to undertake a pyrogenicity test following the same pharmacopeial method but with animals that respond quite differently. In the case of routinely testing batches of vaccine, which have already been tested by the manufacturer, at the national control laboratory alignment of testing is crucial as far as possible. However, harmonization between laboratories sited in different countries is impractical if not impossible. Whilst the same species and age of rabbit and, if possible, the same supplier can be employed, the same housing, temperature and food source cannot practically be standardized across facilities in two different countries. This can result in batches of vaccine passing the RPT test in one laboratory and subsequently failing in another.

7 Measuring the maximum febrile response

Febrile reactions are common adverse events observed following immunization, but the time at which this fever is evident varies between vaccines (Copeland et al., 2005; Tapiainen and Heininger, 2005). Therefore, the fever response in the rabbit should be assessed for each specific vaccine to ensure the maximum fever response is captured within the assay. Indeed a study that addressed a similar concern, i.e., whether measuring the temperature of rabbits in pre-clinical toxicology studies between 2-6 hours according to standard GLP practice is optimal for OMV vaccines, supports the proposal that product specific models may be required to capture the maximum fever response (Kaaijk et al., 2013a). The current EP, USP and JP pyrogen tests administer the sample and monitor the rabbits' temperatures for 3 hours. However, results of an unpublished study aiming to harmonize a rabbit pyrogen test method between laboratories provide further evidence that the time needed to capture the maximum fever response to OMVs in rabbits may require optimization. In Figure 1, the rabbit fever responses to purified endotoxin, derived from *Escherichia coli*, at different concentrations are shown. In Figure 2 the rabbit fever responses to vaccines containing OMVs are depicted. The peak in temperature rise in response to purified endotoxin is captured between 1 and 2 hours for all doses and a decrease in body temperature can be seen between 2-6 hours. In contrast, the maximum temperature rise recorded in rabbits following administration of a vaccine containing meningococcal OMVs was at 4.5 hours; due to ethical restrictions this experiment was not continued to allow the recovery of temperature to baseline.

These data suggest that the response to endotoxin alone is different from the response when it is presented to the innate immune system as part of an OMV. Furthermore it suggests the pharmacopeial methods are not suitable for testing vaccines like OMVs, which intrinsically contain endotoxin.

8 Monocyte Activation Test

The monocyte activation test (MAT) was first conceived as an alternative to the BET and RPT by Poole et al. in the 1980s (Poole et al., 1988). It uses human derived monocytes to mimic the fever response to pyrogens *in vivo* and arguably best represents the situation encountered when medicines are administered to humans. Monocytes are involved in the innate immune response and express toll-like receptors (TLRs) that bind pyrogens. Binding of pyrogens to the receptors stimulates release of pro-inflammatory cytokines (e.g., IL-6, IL-1β or TNFα) that can be measured. The amount of cytokine released is a measure of the pyrogenicity of a substance (Hermanns et al., 2012;

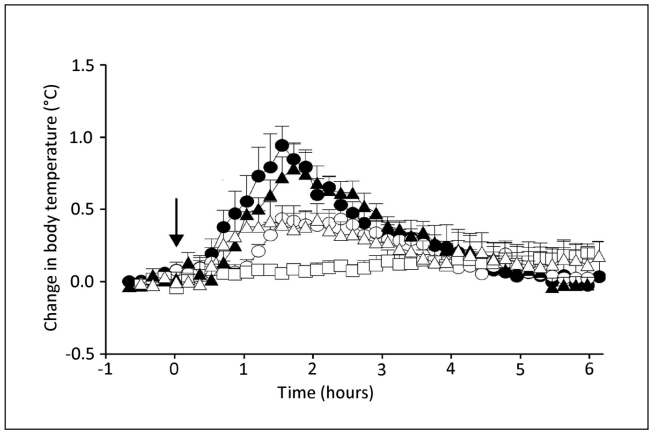

Fig. 1: Temperature change in rabbits following administration of saline or endotoxin
□ saline, ○ 20 IU/kg, △ 30 IU/kg, ▲ 40 IU/kg and ● 80 IU/kg of endotoxin. The temperatures were taken rectally according to EP and USP pyrogen test methods.

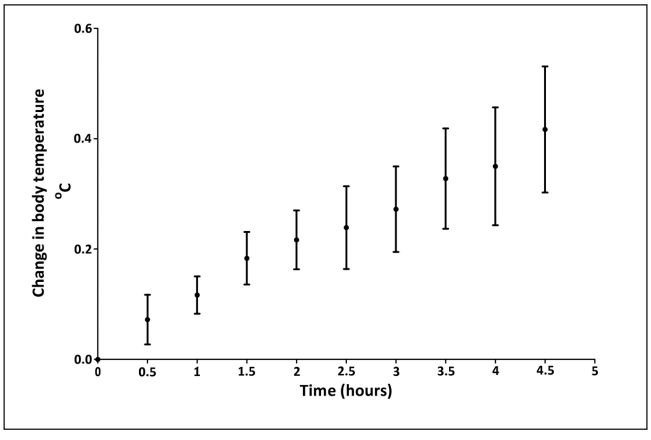

Fig. 2: Temperature changes in rabbits following administration of vaccine containing meningococcal OMVs
The temperatures were taken rectally according to EP and USP pyrogen test methods.

Poole et al., 1988). The MAT became a European Pharmacopeial method in 2010 (EP 2.6.30). The monograph describes three methods and whole blood, peripheral blood mononuclear cells or cell lines can be used for any of these methods. MATs have been used to evaluate meningococcal OMV based vaccines (Kaaijk et al., 2013b; Stoddard et al., 2010); one of these studies used whole blood and the other the monocytic cell line MM6. The pyrogen content of the vaccines was assessed via IL-6 release in both assays and in addition TNF-α was also measured in one study. NIBSC have used this assay since the late 1980's to assess various products (Gaines Das et al., 2004; Poole et al., 1988) and the assay has recently been modified for evaluating vaccines containing meningococcal OMVs. It provides a quantitative readout allowing the consistency of batches of vaccine to be assessed and gives statistical confidence that batches of vaccine are not more pyrogenic than those found to be safe in clinical trials.

9 Conclusion

The RPT was developed and utilized during a period of time when there was a major problem with the sterility of intravenously administered biologicals. The test improved the quality of the products, reducing the adverse reactions seen prior to its implementation. For detecting contamination in a product that should be sterile this method offered obvious benefits and remained the industry standard method for pyrogen testing until the introduction of the BET twenty years later. The first developmental meningococcal OMV vaccines were tested by the RPT partly because they were produced before the BET was established as a pharmacopeial method. The RPT has remained a quality control test on the basis that it can detect non-endotoxin pyrogens. However, the high levels of endotoxin in OMV based vaccines mean that if the test is performed as described in the pharmacopeia, the rabbits have an inflammatory response and the vaccine fails the test. To overcome this, the vaccine is first diluted up to 700 fold prior to administration to the rabbit and the test can no longer be considered a safety test. In addition, there is evidence that the method requires optimizing to ensure the maximum fever response is captured. The limitations of the assay are widely recognized and thus the RPT is considered to be a consistency rather than a safety test. The use of the consistency approach for the quality control of vaccines is underpinned by strict application of GMP verified by in process and final product testing to confirm subsequent batches are consistent with those shown to be safe and efficacious in clinical trials by the manufacturer (De Mattia al., 2011). The tests used to confirm this consistency include physicochemical, immunochemical and *in vitro* bioassays (Kulpa-Eddy and Dusek, 2011). Moreover the approach aims to replace *in vivo* tests based on their inherent variability and for ethical reasons. Thus, as a consistency test, the RPT is flawed as it provides qualitative rather than quantitative data making comparison between lots limited. Furthermore, it counters the principles of the 3Rs (reduce, refine replace) tenet, primarily because it is not ethical to use animals in an experiment which provides no meaningful data.

In conclusion, the use of the RPT for evaluating vaccines known to be pyrogenic is questionable and both manufacturers and national control laboratories need to consider carefully what information can be gained from running the test. Looking to the future, vaccines are becoming increasingly complex with multiple components along with the development of novel adjuvants designed to evoke the innate immune response. Thus it is likely that the RPT will be unsuitable for testing these new vaccines. The MAT is suggested as an alternative method providing quantitative data in a system which measures human inflammatory responses. This is in line with a move to the consistency approach for ensuring safety, efficacy and quality of vaccines which, if properly implemented, could make the use of animal tests for routine testing by national control laboratories minimal or even non-existent.

References

Bang, F. B. (1956). A bacterial disease of Limulus polyphemus. *Bull Johns Hopkins Hosp 98*, 325-351.

Bellentani, L. (1982). Cyclic and chronobiological considerations when employing the rabbit fever test. *Prog Clin Biol Res 93*, 329-342.

Bjune, G., Hoiby, E. A., Gronnesby, J. K. et al. (1991). Effect of outer membrane vesicle vaccine against group B meningococcal disease in Norway. *Lancet 338*, 1093-1096. http://dx.doi.org/10.1016/0140-6736(91)91961-S

Copeland, S., Warren, H. S., Lowry, S. F. et al. (2005). Acute inflammatory response to endotoxin in mice and humans. *Clin Diagn Lab Immunol 12*, 60-67. http://dx.doi.org/10.1128/cdli.12.1.60-67.2005

de Moraes, J. C., Perkins, B. A., Camargo, M. C. et al. (1992). Protective efficacy of a serogroup B meningococcal vaccine in Sao Paulo, Brazil. *Lancet 340*, 1074-1078. http://dx.doi.org/10.1016/0140-6736(92)93086-3

De Mattia, F., Chapsal, J. M., Descamps, J. et al. (2011). The consistency approach for quality control of vaccines – a strategy to improve quality control and implement 3Rs. *Biologicals 39*, 59-65. http://dx.doi.org/10.1016/j.biologicals.2010.12.001

European Pharmacopoeia. (2005). Meningococcal polysaccharide vaccine. *8th edition (8.7)*, 5669-5670.

European Pharmacopoeia. (2008). 2.6.9. Abnormal Toxicity. *8th edition (8.7)*, 184-184.

Ferrari, G., Garaguso, I., Adu-Bobie, J. et al. (2006). Outer membrane vesicles from group B Neisseria meningitidis delta gna33 mutant: Proteomic and immunological comparison with detergent-derived outer membrane vesicles. *Proteomics 6*, 1856-1866. http://dx.doi.org/10.1002/pmic.200500164

Fisseha, M., Chen, P., Brandt, B. et al. (2005). Characterization of native outer membrane vesicles from lpxL mutant strains of Neisseria meningitidis for use in parenteral vaccination. *Infect Immun 73*, 4070-4080. http://dx.doi.org/10.1128/IAI.73.7.4070-4080.2005

Frasch, C. E. and Peppler, M. S. (1982). Protection against

group B Neisseria meningitidis disease: Preparation of soluble protein and protein-polysaccharide immunogens. *Infect Immun 37*, 271-280.

Frasch, C. E., van Alphen, L., Holst, J. et al. (2001). Outer membrane protein vesicle vaccines for meningococcal disease. *Methods Mol Med 66*, 81-107. http://dx.doi.org/10.1385/1-59259-148-5:81

Fredriksen, J. H., Rosenqvist, E., Wedege, E. et al. (1991). Production, characterization and control of MenB-vaccine "Folkehelsa": An outer membrane vesicle vaccine against group B meningococcal disease. *NIPH Ann 14*, 67-79; discussion 79-80.

Gaines Das, R. E., Brügger, P, Patel, M. et al. (2004). Monocyte activation test for pro-inflammatory and pyrogenic contaminants of parenteral drugs: Test design and data analysis. *J Immunol Meth 288*, 165-177. http://dx.doi.org/10.1016/j.jim.2004.03.002

Goldschneider, I., Gotschlich, E. C. and Artenstein, M. S. (1969a). Human immunity to the meningococcus. I. The role of humoral antibodies. *J Exp Med 129*, 1307-1326. http://dx.doi.org/10.1084/jem.129.6.1307

Goldschneider, I., Gotschlich, E. C. and Artenstein, M. S. (1969b). Human immunity to the meningococcus. II. Development of natural immunity. *J Exp Med 129*, 1327-1348. http://dx.doi.org/10.1084/jem.129.6.1327

Greisman, S. E. and Hornick, R. B. (1969). Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin. *Proc Soc Exp Biol Med 131*, 1154-1158. http://dx.doi.org/10.3181/00379727-131-34059

Hartung, T., Aaberge, I., Berthold, S. et al. (2001). Novel pyrogen tests based on the human fever reaction. The report and recommendations of ECVAM Workshop 43. European Centre for the Validation of Alternative Methods. *Altern Lab Anim 29*, 99-123.

Hasiwa, N., Daneshian, M., Bruegger, P. et al. (2013). Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test. *ALTEX 30*, 169-208. http://dx.doi.org/10.14573/altex.2013.2.169

Hermanns, J., Bache, C., Becker, B. et al. (2012). Alternatives to animal use for the LAL-Assay. *ALTEX Proc 1*, 81-84. http://www.altex.ch/resources/081084_Spreitzer31.pdf

Hoffmann, S., Luderitz-Puchel, U., Montag, T. et al. (2005a). Optimisation of pyrogen testing in parenterals according to different pharmacopoeias by probabilistic modelling. *J Endotoxin Res 11*, 25-31. http://dx.doi.org/10.1179/096805105225006678

Hoffmann, S., Peterbauer, A., Schindler, S. et al. (2005b). International validation of novel pyrogen tests based on human monocytoid cells. *J Immunol Methods 298*, 161-173. http://dx.doi.org/10.1016/j.jim.2005.01.010

Hort, E. C. and Penfold, W. J. (1912). Microorganisms and their relation to fever: Preliminary communication. *J Hyg (Lond) 12*, 361-390. http://dx.doi.org/10.1017/S0022172400005052

Hull, D., McIntyre, J. and Vinter, J. (1993). Age-related changes in endotoxin sensitivity and the febrile response of newborn rabbits. *Biol Neonate 63*, 370-379. http://dx.doi.org/10.1159/000243957

Kaaijk, P., van der Ark, A. A., van Amerongen, G. et al. (2013a). Nonclinical vaccine safety evaluation: Advantages of continuous temperature monitoring using abdominally implanted data loggers. *J Appl Toxicol 33*, 521-526. http://dx.doi.org/10.1002/jat.2720

Kaaijk, P., van Straaten, I., van de Waterbeemd, B. et al. (2013b). Preclinical safety and immunogenicity evaluation of a nonavalent PorA native outer membrane vesicle vaccine against serogroup B meningococcal disease. *Vaccine 31*, 1065-1071. http://dx.doi.org/10.1016/j.vaccine.2012.12.031

Kulpa-Eddy, J. and Dusek, D. (2011). Application of the consistency approach to reduce animal use in vaccine potency testing. *Procedia in Vaccinology 5*, 232-235. http://dx.doi.org/10.1016/j.provac.2011.10.024

Kuronen, T., Peltola, H., Nors, T. et al. (1977). Adverse reactions and endotoxin content of polysaccharide vaccines. *Dev Biol Stand 34*, 117-125.

Martin, W. J. and Marcus, S. (1964). Studies on Bacterial Pyrogenicity. 3. Specificity of the United States Pharmacopeia Rabbit Pyrogen Test. *Appl Microbiol 12*, 483-486.

Medsafe (2006). Full Consent Application for the Meningococcal GroupB OMV vaccine MeNZB, by Chiron at the Rosia nndd Siena Sites, Italy Application in Accordance with Medicines Act 1981 and Medicines Regulations 1984, for Consent to distribute in New Zealand.

Oster, P., Lennon, D., O'Hallahan, J. et al. (2005). MeNZB: A safe and highly immunogenic tailor-made vaccine against the New Zealand Neisseria meningitidis serogroup B disease epidemic strain. *Vaccine 23*, 2191-2196. http://dx.doi.org/10.1016/j.vaccine.2005.01.063

Poole, S., Thorpe, R., Meager, A. et al. (1988). Detection of pyrogen by cytokine release. *Lancet 1*, 130. http://dx.doi.org/10.1016/S0140-6736(88)90338-8

Roberts, K. J. (2007). The Pyrogen Test. In K. L. Williams (ed.), *Endotoxins – Pyrogens, LAL Testing and Depyrogenation* (261-271). CRC Press.

Seibert, F. B. (1925). The cause of many febrile reactions following intravenous injections. I. *American Journal of Physiology – Legacy Content 71*, 621-651.

Sierra, G. V., Campa, H. C., Varcacel, N. M. et al. (1991). Vaccine against group B Neisseria meningitidis: Protection trial and mass vaccination results in Cuba. *NIPH Ann 14*, 195-207; discussion 208-110.

Stoddard, M. B., Pinto, V., Keiser, P. B. et al. (2010). Evaluation of a whole-blood cytokine release assay for use in measuring endotoxin activity of group B Neisseria meningitidis vaccines made from lipid A acylation mutants. *Clin Vaccine Immunol 17*, 98-107. http://dx.doi.org/10.1128/CVI.00342-09

Tapiainen, T. and Heininger, U. (2005). Fever following immunization. *Expert Rev Vaccines 4*, 419-427. http://dx.doi.org/10.1586/14760584.4.3.419

Tsai, C. M., Frasch, C. E., Rivera, E. et al. (1989). Measurements of lipopolysaccharide (endotoxin) in meningococcal protein and polysaccharide preparations for vaccine usage. *J Biol Stand 17*, 249-258. http://dx.doi.org/10.1016/0092-1157(89)90017-6

van Dijck, P. and van de Voorde, H. (1977). Factors affecting pyrogen testing in rabbits. *Dev Biol Stand 34*, 57-63.

Vipond, C., Suker, J., Jones, C. et al. (2006). Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared from the group B strain NZ98/254. *Proteomics 6*, 3400-3413. http://dx.doi.org/10.1002/pmic.200500821

Williams, K. L. (2007). Endotoxin Relevance and Control Overview. In K. L. Williams (ed.), *Endotoxins – Pyrogens, LAL Testing and Depyrogenation* (27-45). CRC Press.

World Health Organization (1976). Requirements for meningococcal polysaccharide vaccine (requirements for biological substances no. 23). *WHO Technical Report Series 594*, 72-73

World Health Organization (2013). Methods of Analysis: 3. Biological methods: 3.4 Test for bacterial endotoxins. 5th edition, 2015.

Zollinger, W. D., Mandrell, R. E., Altieri, P. et al. (1978). Safety and immunogenicity of a Neisseria meningitidis type 2 protein vaccine in animals and humans. *J Infect Dis 137*, 728-739. http://dx.doi.org/10.1093/infdis/137.6.728

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgement

This work was funded, in part, by a grant from UK Department of Health's Policy Research Programme, Grant Number 044/0069.

Correspondence to

Caroline Vipond
Department of Bacteriology
National Institute of Biological Standards and Control
South Mimms
Potter Bar
EN6 3QG, UK
Phone: +44 1707 641000
e-mail: caroline.vipond@nibsc.org