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Dear Dr Stokes,

re: Independent Scientific Peer Review Meeting on the Use of *In vitro* Pyrogenicity Testing Methods, Bethesda, MD, Feb 6th 2007 – request for comments.

In accordance with the invitation issued 12th Dec 2006, we would like to submit some comments for your consideration, specifically to the document 'Draft ICCVAM Test Method Recommendations: I*n Vitro Pyrogenicity Test Methods*', dated 01 Dec 2006 (file PWGrec12016.pdf).

We submit these comments as independent developers of an alternative proprietary *in vitro* pyrogen test, or IVPT. The test has been developed by us at the Faculty of Pharmaceutical Sciences at the University of Copenhagen [1]. Our test differs from the five ECVAM 'interleukin' tests under consideration here in that it is based on the measurement of reactive oxygen species produced from terminally-differentiated cells derived from the human HL-60 promyelocytic leukemia cell line. Whilst we believe that our test has all the advantages claimed by the various ECVAM test methods over the RPT, and more besides, our comments here will be restricted to the ICCVAM evaluation of the validation status of these ECVAM tests and the draft recommendations for such test methods.

Comments to PWGrec12016:

1.1 Draft recommended test method uses

"While the scientific basis of these (ECVAM) test methods suggests that they have the capability to detect pyrogenicity produced by a wider range of pyrogens (i.e. those mediated by non-endotoxin sources), there is insufficient data to support this broader application."

It is very clear from the current literature, and indeed from our own experience of many years working with similar assays (PBMC/IL-1 and MM6/IL-6 assays), that of the five ECVAM tests under evaluation, only the MonoMac6 test has a relevant and useful sensitivity towards non-endotoxin pyrogens. However, this property of the MonoMac6 test does not yet appear to have been validated.
Since the aim of your evaluation is to find an appropriate replacement for the RPT, and that one of the principal strengths of the RPT is that it offers the possibility of detecting pyrogens that would otherwise be missed by the BET, we offer the comment that perhaps it should be considered essential that a suitable IVPT replacement for the RPT must be validated in respect of its ability to detect relevant non-endotoxin pyrogens.

1.2 Draft recommended Future Studies

We wholeheartedly agree with the recommendation that "additional studies that include a broader range of pyrogenic materials…" be conducted if any of the five test methods under consideration are to be considered as potential replacements for the RPT.
We also strongly agree with footnote (3), that "an international standard [for non-endotoxin pyrogens]" is needed in order to demonstrate the utility of these (and other) test methods for the detection of non-endotoxin pyrogens. We suggest that suitable sources of non-endotoxin standards for this purpose might include yeast, fungi and gram-positive bacteria e.g. *Candida albicans* and *Staphylococcus aureus* either as whole organisms or isolated components hereof as for instance LTA from *S. aureus*. We suggest these two because both pathogens are of clinical relevance.

Appendix A, 1.4.4: Similarities and Differences in the Endpoints of IPT Methods and Currently recognized Pyrogenicity Test Methods

"…the *in vitro* release of pro-inflammatory cytokines, such as IL-1β and IL-6, is intended to predict the onset of [an inflammatory response]"
Although we do not argue against the relevance of these endpoints *per se*, we feel that we must make the comment that simple serum-level increases in either one or both of these interleukins are not sufficient in themselves to predict either an inflammatory reaction or a febrile response [2]. We should also like to point out that, although the focus here is on production of interleukins in the tests being evaluated, there are other endpoints that are just as relevant for prediction of inflammatory responses by the human immune system, indeed perhaps more so, and that one of these is the production of reactive oxygen species by macrophage- and PMN-like cells when challenged with pyrogenic materials.

Appendix A, 2.3.1: Essential Test method Components, *In Vitro* Cell Culture Conditions

Regarding the use of cryo-preserved whole blood, we appreciate that this is one possible way to avoid the need to make large numbers of willing blood donors available to testing laboratories. However, several laboratories, including our own, have experienced significant problems using cryo-preserved blood in these assays – in our case, the "cryo WB/IL-1" test, commercially obtained from Charles River Labs. Whilst the WB/IL-1 test delivered the results expected using fresh whole human blood, when we tested the same kit with cryo-preserved blood obtained from a source recommended by the manufacturers, it gave no results at all. We believe that the reason for this was that the cryo-preserved blood cells had been irretrievably damaged by the freezing process; the blood sample, thawed according to instructions, was thick and denatured with every indication of extreme cellular damage. From our discussions with others who have also tried using cryo-preserved blood in this test, we conclude that this is a not un-common problem.

Appendix A, 2.3.3.2: Positive Control Substance:

An important distinction between the BET/LAL test and the RPT is that the BET detects only endotoxin pyrogens, whereas the rabbit pyrogen test is capable of also detecting non-endotoxin pyrogens. We suggest that it should therefore be a requirement of the performance standards for any IVPT that might replace the RPT that said *in vitro* test is assessed directly for its ability to detect non-endotoxin pyrogens, as well as LPS.
We therefore suggest that the performance standards include a requirement for one or more positive control pyrogenic substances selected from a group of non-endotoxin pyrogens (perhaps those suggested in our comment to point 1.2, above), in addition to the reference standard LPS to demonstrate adequate sensitivity of the cell system to relevant pyrogens. The sensitivity of any suitable test method to these non-endotoxin pyrogens should be at least comparable to the sensitivity of the rabbit pyrogen test to these same substances.

Appendix A, 2.4: Reference Substances for In Vitro Pyrogenicity Test Methods

In line with the various comments made above, we would suggest that Reference Substances be spiked not only with Gram-negative endotoxin standards, but also non-endotoxin pyrogen standards in order to properly assess the accuracy and reliability of a proposed IVPT that should replace the RPT.

We hope that these few comments will be useful to you in the process of evaluating the validation status of the EVCAM tests, and for drafting future Performance Standards by which to determine the relevance and reliability of these and other *in vitro* test methods for the highly desirable purpose of replacing the RPT.

Yours sincerely,

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P.S. In case this may be of interest, we have attached the most recent results obtained with our HL-60 ROS IVPT, further optimized from the test reported in [1]. The table reports the responses obtained from a wide variety of pyrogenic components. This table also contains results obtained by us for these same substances tested using the WB/IL-1 IPT (Charles River Labs), and literature data for the same substances run in the RPT.

References:

[1] Timm, M., Hansen, E.W., Moesby, L., Christensen, J.D. (2006). Utilization of the human cell line HL-60 for chemiluminescence based detection of microorganisms and related substances. *Eur J Pharmaceutical Sciences* **27**: 252-258

[2] Blatteis, C.M. (2006). Endotoxic fever: New concepts of its regulation suggest new approaches to its management. *Pharmacology & Therapeutics* **111**: 194 – 223.

Pyrogen Test Benchmark Data: Hansen & Timm, University of Copenhagen

Positive detections by four assays evaluated for pyrogen determination

Sample	HL-60 assay	IPT assay	Rabbit pyrogen test	LAL test
Zymosan 0,5 µg/ml	+	-	-	-
Zymosan 5 µg/ml	+	+	-	-
LTA standard (0,5 EEU/ml)	+	+		-
Candida albicans 10^4 yeasts/ml	+	-	-	-
Candida albicans 10^5 yeasts/ml	+	-	-	-
Saccharomyces cerevisiae 10^4 yeasts/ml	+	-	-	-
Saccharomyces cerevisiae 10^5 yeasts/ml	+	-	-	-
LTA from *Bacillus subtilis* 25 ng/ml	+	(-)	-*	-
LTA from *Bacillus subtilis* 100 ng/ml	+	+	-*	-
Staphylococcus aureus 10^5 bacteria/ml	+	-	-*	-
Staphylococcus aureus 10^6 bacteria/ml	+	-	+*	-
Bacillus subtilis 10^4 bacteria/ml	+	+	+*	-
Bacillus subtilis 10^5 bacteria/ml	+	+	+*	-
Salmonella typhimurium 10^3 bacteria/ml	+	(-)	-*	+
Salmonella typhimurium 10^4 bacteria/ml	+	+	+*	+
Aspergillus niger spores 10^5 spores/ml	+	-		-
Aspergillus niger spores 10^6 spores/ml	+	-		-
LPS standard 5 EU/ml	+	+	+*	+
LPS standard 2,5 EU/ml	+	+	+*	+
LPS standard 1 EU/ml	+	+	+*	+
LPS standard 0,5 EU/ml	+	+	+*	+
LPS standard 0,25 EU/ml	+	-	-*	+
LPS standard 0,125 EU/ml	+	-	-*	+

(-) samples do excite a response above non stimulated control, but do not score as pyrogenic according to manufactures description.

(*) data obtained from literature.