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Dr. William Stokes
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Dear Dr. Stokes:

Please, find below our comments to the "Independent Peer Review Panel Report: Five *In vitro* Test Methods Proposal for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products".

Comment to A 1.2.1 Criterion 4 (page 3), also comment to A 4.6 (page 12)

The PRP discussed critically whether the right end-points had been set in the validation study regarding sensitivity (respectively detection limits) of the tests. We are wondering why the PRP did not consider the internationally accepted endotoxin limits. They are regulated in the respective monographs for endotoxin testing (Bacterial Endotoxin Test, BET) since decades in the international pharmacopoeias. The endotoxin limit for parenteral drugs intended for intravenous administration is 5 International Units endotoxin (E.U., to calibrate using the WHO Endotoxin Standard which is identical with the US Endotoxin Standard) per kilogram body weight of the patient (in the past: administration during one hour period; following the current ICH document: as a bolus injection). Exactly this endotoxin limit had been used for calculation of the detection limits in examining the involved drugs. As usual in pyrogen testing, a patient having a body weight of 70 kg (corresponding to a maximal endotoxin content of 350 E.U. of the whole volume of the given drug) had been considered for calculation. Furthermore, the WHO Endotoxin Standard had been used in the study.

Additionally, the sensitivity respectively the detection limit of Rabbit Pyrogen Test (RPT) had been considered in the study design. The sensitivity of RPT can be calculated considering the fever threshold respectively the threshold of significant temperature increase of rabbits. The most sensitive rabbit strains show a fever

threshold of 5 E.U. per kilogram body weight (see papers Hoffmann et al. 2005, Journal of Immunological Methods, Vol. 298, pp. 161-173, and Hoffmann et al. 2005, Journal of Endotoxin Research, Vol. 11, pp. 1-7). This endotoxin concentration may be contained in maximally 10 milliliter which represents the highest allowed burden for the rabbits following the animal protection lows. In consequence, the sensitivity of RPT is represented by 0.5 E.U. per milliliter (5 E.U. in 10 ml = 0.5 E.U. per ml) corresponding to 50 pg/ml. This endotoxin concentration had been used for setting the detection limits of the five *In vitro* Test methods and it is (at least) fulfilled by all tests. So the five alternative pyrogen tests meet worst case conditions of RPT and guarantee, therefore, a high safety level for the patients.

It has to be mentioned here that the endotoxin limit regulation mentioned above (5 E.U. per kg body weight of the recipient) comes directly from rabbit's sensitivity. Preparing the implementation of BET into the pharmacopoeias decades ago, the safety level of the drugs was the most important criterion. In this time, only data from the rabbit were available and, consequently, they were used for definition of endotoxin limits. This was a wise decision since the fever threshold of human beings lies in a range of 10 - 20 E.U. per kg body weight and, therefore, the safety of drugs regarding potential pyrogenicity is guaranteed. Taking into account the background of endotoxin limits, it is surprising when the expert panel used the phrasing "theoretical sensitivity" of the RPT. As demonstrated above, the calculations for the validation study reflects exactly the current practice and the regulatory use.

Comment to A 1.2.1 Criterion 5 (page 3)

The PRP stated: "The new test methods clearly take longer to produce definitive results". This statement does not consider the mandatory pre-test for RPT which has to be performed two days prior to the main test employing the same animals (i.e. RPT lasts all together not less than 48 hours). The *in vitro* tests are usually performed within less than 20 hours (i.e. incubation of the cells overnight and measuring the cytokine content in ELISA next morning). If necessary, the tests can be performed within 10 hours by shortening the cell culture to 6 hours.

Comment to A 1.2.2 (page 3)

There is a contradiction in this passage. On the one, hand it is stated: "The RPT (Rabbit Pyrogen Test) detects both endotoxin and non-endotoxin pyrogens, but the *in vitro* pyrogen tests have not been validated for non-endotoxin pyrogens. Therefore, they cannot be considered complete replacements for the RPT." On the other hand, it is stated: "The BET (Bacterial Endotoxin Test) detects endotoxin in most cases and is used instead of the RPT for this purpose." It is not understandable why the *in vitro* tests, able to detect endotoxin, cannot replace the RPT but BET, able to detect only endotoxin, can.

There is a clear need for tests able to detect non-endotoxin pyrogens (for examples regarding adverse reactions caused by non-endotoxin pyrogens, see comment to A 4.0 below, please). The PRP mentioned several times in the report that non-endotoxin pyrogens were not included in the validation study. This holds true but

there is a broad specter of publications demonstrating that *in vitro* pyrogen test methods are able to detect non-endotoxin pyrogens. This includes papers which applied the same procedure for pyrogen testing using human whole blood as used in the validation study (e.g. Hermann et al. European Journal of Immunology, 2002, Vol. 32, pp. 541-551, and Morath et al., Infection and Immunity, 2002, Vol. 70, pp. 938-944). One would appreciate if the PRP (at least) had mentioned those publications.

Comment to A 1.3.2 (page 4)

The PRP stated: "A major concern is the lack of validation of these new assays directly compared to the RPT." There were data available on several studies regarding comparison of RPT and *in vitro* pyrogen tests as used in the validation study. The first study (Spreitzer at al., Altex, 2002, Vol. 19, pp. 73-75) concerns a comparative study of Rabbit Pyrogen Test and Human Whole Blood Assay implementing 29 batches of 10 different Human Serum Albumins from 5 manufacturers. All together, 261 rabbits were included in the study. Two endotoxin spike concentrations in the range of RPT detection limit were used. There was no failure in the *in vitro* pyrogen test. Actually, the *in vitro* test appeared more sensitive than the RPT. In the second study (Andrale et al. International Journal of Pharmaceutics, 2003, Vol. 265, pp. 115-124) a broad range of parenterals (15 different drugs) were tested comparing RPT and BET with Human Whole Blood Assay and, additionally, with Human Peripheral Blood Mononuclear Cell (PBMC) Test. The two *in vitro* tests showed good agreement overall, both with each other and with BET and the RPT. The third study concerns a comparison of six different Coagulation Factor VIII Concentrates (3 lots each) in RPT and in Human Whole Blood Assay. 162 rabbits were included in the study; two different endotoxin spikes in the range of RPT detection limit were used. As in the above mentioned albumin study, no failure was seen in the *in vitro* assay. Again, the *in vitro* test appeared more sensitive as the RPT. The latter study is not published yet but, due to our knowledge, the data had been provided to the PRP.

Comment to A 3.1 (page 9)

The PRP stated: "No 'classical' examples of biological products or medical devices were included; thus, the validation of either of these categories has not been provided."

It should be mentioned that Coagulation Factor VIII concentrate had been included in the pre-validation study where it was successfully tested. This preparation could not be considered in main study because of its high price. Additionally, see the above comment to A 1.3.2, please. Human Serum Albumin and Coagulation Factor VIII concentrate belong to the 'classical' biological products.

Comment to A 3.4 (page 10)

The PRP stated: "The coding procedures were adequate for the assessment of relevance during the validation studies. However, the identity of substances used in the reproducibility analyses was not blinded (although the spike concentrations were). A reason was not given."

It is commonly known that a pharmaceutical company has to perform for any test the so called product validation in order to exclude potential interferences of the preparation with the test system (e.g. inhibition of the test by the drug). Of course, the best approach is to perform the product validation using a clean batch of the product. The latter procedure had been chosen for the validation study considering the practice in pharmaceutical industry.

Comment to A 4.0 (page 10)

The PRP stated: "… a summary of reference data demonstrating whether substances that were shown to be pyrogenic in humans either passed or failed the RPT, BET, or in vitro tests would have been useful." There are data published regarding adverse reactions (fever respectively pro-inflammatory reactions) in patients caused by drugs which were negative in RPT as well as in BET, but could be tested positive using *in vitro* pyrogen tests. The first event happened with a Tick Borne Encephalitis Vaccine which induced fever up to cramps and hospitalization in around 50 percent of the recipients. As mentioned above, both RPT and BET remained negative in testing the product. In contrast, this product produced positive results with blood samples of 50 percent of the donors applying the *in vitro* pyrogen test (Whole Blood Test, Fischer et al., Altex, 2001, Vol. 18, pp. 47-49). Another example concerns a dialysis solution which caused aseptic peritonitis in the patients (Martis et al. Lancet, 2005, Vol. 365, pp. 588-594). Again, both RPT and BET were negative whereas the *in vitro* pyrogen test (PBMC Test) could identify the incriminated batches. These two examples demonstrate that the in vitro pyrogen tests are in certain cases superior to the RPT since they are working in the 'homologous system' (i.e. human indicator cells and fever/pro-inflammatory reactions in humans).

It should be pointed out that the PRP should know the above cited cases as one of its members was in touch with both of them.

Comment to A 4.3 (page 11)

The PRP stated: "Archived records have not been audited by ECVAM or ICCVAM."

This statement is wrong as the archived records have been audited by ECVAM in PEI.

Comment to A 4.4 (page 11)

The PRP stated: "However, the PEI did not have formal GLP accreditation (refer to Section 5.5, ECVAM request for additional information)."

As the Federal Agency of Sera and Vaccines, the unit for pyrogen and endotoxin testing of the Paul Ehrlich Institute (PEI) is accredited following ISO 17025 (for the lists of accredited methods in PEI see http://www.pei.de/cln_049/nn_162948/EN/infos-en/pu-en/11-quality-management-en/accredited-methods-en/akkreditierungen-inhalt-en.html, please).

Despite the above cited ECVAM answer, it should be mentioned here that one of the PRP members visited the PEI unit for pyrogen and endotoxin testing and knows its accreditation status.

Comment to A 5.1.1 (page 12)

The PRP stated: "Quality control (QC) testing of cell viability is not performed. Viability testing of human cells before and after incubation should be performed."

This statement does not consider how the tests are designed. It is one of the advantages of the *in vitro* pyrogen tests that additional testing of cell viability is not necessary. The functionality of cells is controlled in every test via reaction of monocytes to endotoxin controls which have to induce a defined minimum of cytokine concentration. This internal quality control gives more information on the status of the monocytes than a viability test; viability test indicates only that the cells are living whereas functionality test indicates that cells are living and able to react.

Comment to A 10.2 (page 21)

The PRP stated: "The discussion that reduction of the use of animals (i.e., rabbits) will be associated with the increased use of another animal (i.e., humans) is inadequate."

This statement seems to be far away from practice. It is commonly known that worldwide millions of people are donating blood (for example, more than 5 millions blood donations per year in Germany). One whole blood donation consists of 500 ml blood, a volume which would be theoretically sufficed for 5,000 to 50,000 whole blood pyrogen tests. Therefore, the use of human blood for pyrogen testing would lead to a marginal increase of blood donation. Blood donors are mainly volunteers offering their blood for philanthropic reasons. Donating blood for safety testing of drugs for human use is a philanthropic attitude, too.

Comment to A 11.4 (page 22)

The PRP stated: "Furthermore, the *in vitro* pyrogen test methods are dependent on the availability of donors or blood supplies, which might further restrict the frequency of which these tests can be performed."

This statement does not consider the cryo-preserved blood since it would be available at any time.

Thank you for your attention to these comments.

Sincerely,

Dr. Thomas Montag-Lessing
Dr. Ingo Spreitzer