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Dr. William Stokes
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Via electronic transmission to: niceatm@niehs.nih.gov

Dear Dr. Stokes:

These comments are submitted on behalf of People for the Ethical Treatment of Animals, Humane Society of the United States, Physicians Committee for Responsible Medicine, and the Alternatives Research & Development Foundation, a coalition of animal protection, alternatives development, and health advocacy organizations representing more than 10 million Americans in response to a December 16, 2005 notice in the *Federal Register* inviting public comment on a proposed peer review panel evaluation of five human biology-based *in vitro* pyrogenicity test methods. We consider these methods to have great potential to replace the existing animal-based methods and we appreciate the work that has gone into the development of the background review documents (BRDs) by the European Center for the Validation of Alternative Methods (ECVAM) and into their preliminary review by Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).

We believe that an international peer review of these novel pyrogenicity test methods is appropriate, necessary, and should be given extremely high priority. A thorough yet expeditious review of these tests by an expert panel resulting in the endorsement of at least one proposed test method should be viewed as a potential quick win in the efforts of ICCVAM to meet its statutory mandate to promote the replacement, reduction, or refinement of animal-based testing (42 *U.S.C.* Sec. 2851-3(b)).

Need for Speedy Review of Novel Pyrogenicity Tests

Pyrogenicity testing is most commonly used to ensure that medical treatments (particularly injectable medicines or implanted devices) are free of fever-inducing contaminants. Currently used methods of animal-based pyrogenicity testing have significant scientific and practical limitations (described below). Human-biology based pyrogenicity tests are more sensitive, more consistent, and more versatile, but most importantly, more accurate. Since they are based on human immune system responses, they represent the most relevant and best possible means of predicting human pyrogenic potential. Swift validation of the proposed *in vitro* tests and replacement of animal-based pyrogenicity tests is necessary to best safeguard consumer safety.

Use of the five novel test methods will also better protect the public because they enable testing that was not previously possible due to the limitations of the animal tests. For example, they

enable direct testing of air filters in buildings so that airborne pyrogens can be detected and eliminated; they enable direct testing for pyrogens bound to the surfaces of medical devices, previously not possible; and they enable the testing of cell culture media in order to guarantee its pyrogen-free status. The existing animal-based testing methods are inadequate for testing many important upcoming areas of therapeutics (especially cellular products) which can be tested using the novel human biology-based methods (Hartung et al. *ATLA* 29, 99-123; 2001).

The proposed methods are already in use by over 200 laboratories around the world (EU press release 12/5/03 Reference: IP/03/662) and interest from industry is quite high, thus it is imperative that US federal agencies issue a stance on the validity of these methods. Of the methods under consideration, those utilizing human whole blood (fresh or cryopreserved) and measuring the production of Interleukin-1 are particularly advanced (Methods #2 & 3 in Federal Register notice). These methods have been commercialized as test kits that produce results within a day by the European company Milenia as "PyroCheck" and in the US by Charles River Labs as "Endosafe-IPT." Customers are already using these test kits but cannot stop using the animal-based tests until they know that Agencies will accept their results.

The EU, primarily through ECVAM, has invested considerable resources into the development and international validation of the five submitted *in vitro* test methods with the involvement of over 60 groups from academia, industry, and regulatory bodies. Descriptions of this work have been published in numerous scientific journals. The European Pharmacopeia has installed an international expert group to draft a General Method for these tests and we understand that the ECVAM Scientific Advisory Committee (ESAC) will shortly review the methods and make a statement on their validity. Whereas the vast majority of novel non-animal test methods are validated in the EU years prior to consideration in the US, this submission represents an exciting first opportunity for the US to concurrently evaluate a test method in parallel with the EU. This adds to the imperative that a panel is convened and a review is conducted in a timely manner.

Limitations of Currently Used Pyrogenicity Tests

The considerable limitations of the existing animal-based pyrogenicity tests create another important imperative. The rabbit pyrogenicity test, developed in the 1940s, still consumes an estimated 400,000 rabbits per year (Hartung et al. *ATLA* 30, 49-51; 2002). Animals are locked in full-body restraints while their temperature is monitored through rectal probes and suffer effects which can include fever, breathing problems, organ failure, and fatal shock. Like all animal-based tests, the rabbit pyrogenicity test is time-consuming, costly, and gives results that are species-specific: The potency of pyrogens varies by up to 10,000 in different mammals ((Hartung et al. *ALTEX* 15, 17-18; 1998). However, the rabbit test is scientifically problematic in many additional ways. Even at the highest injected volumes, the detection limit of the rabbit test is above the human fever threshold: humans show a fever response at concentrations as low as 30pg LPS/ml while rabbits' sensitivity varies between 50 and 350 pg LPS/ml. In contrast, the human whole blood IL-1 test has a sensitivity of 10pg LPS/ml (Hartung et al. *ATLA* 29, 99-123; 2001). In addition, the sensitivity of the rabbit test varies depending on the strain, age and gender of rabbit used. Other important problems include the fact that the rabbit test often only gives a

pass/fail, rather than a quantitative, answer; that results are influenced by animal distress as well as seasonal variation; and that inconclusive results necessitating test repetition are common. Lastly, the rabbit pyrogenicity test does not work for many classes of substances including important new therapies such as cellular products or species-specific agents, as well as chemotherapeutics, radiopharmaceuticals, certain biologicals and antibiotics, drugs that cause immune reactions, drugs that influence body temperature such as sedatives/analgesics/anesthetics, and vitamins.

The *in vitro* Limulus Amoebocyte Lysate (LAL) assay, also known as the bacterial endotoxin test or BET, was developed in the 1970s and has largely replaced the rabbit test where possible, but it has severe limitations as well. The most important limitation of the LAL assay is that it only detects endotoxins (components of gram-negative bacteria) but not other pyrogens including gram-positive bacteria, fungi, and viruses. Thus, the LAL assay is used extensively for pharmaceutical testing and for in-process monitoring in biological production but is not suitable as a final release test for complex biologically-derived products that may contain non-endotoxin pyrogens, for material-mediated pyrogenicity, or for substances that chemically or physically interfere with the clotting reaction in the LAL test such as proteins or lipids. It cannot be used for the testing of biological products such as vaccines, immunoglobulins, and clotting factors. In addition, the accuracy of the LAL test for predicting human pyrogens and their potencies is questionable since it is based on the defense system of an arthropod (the coagulation of horseshoe crabs' blood) which is not mechanistically relevant to the human response (Hartung et al. *ALTEX* 15, 9-10; 1998). It is also important to note that the blood used in the LAL assay is obtained by harvesting crabs from the ocean floor and draining ~30% of their blood, which can cause them injury, disrupts their natural life cycles, and depletes their populations, which may make availability of their blood more limited in the future. For ethical and welfare reasons, this test should be replaced as soon as possible.

The rabbit assay is a poor and inadequate test in numerous ways, but the limitations of the LAL assay have led to its continued use. For decades, these tests have been used complementary, but in fact, they are simply limited in different ways and their combined use leaves many gaps in consumer protection and much to be desired. In addition, the two animal-based tests are difficult to correlate with each other. Since the proposed human biology-based tests can detect non-endotoxin pyrogens, they should at the very least completely replace the outdated rabbit pyrogenicity test in final release testing. However, the human biology-based test should also replace the LAL test which should not be conducted if a more humane and relevant human biology-based *in vitro* test is available, which will clearly better safeguard human health.

Human Biology-Based Pyrogenicity Tests

Our understanding of human immunology has advanced rapidly over the last 20 years, and this represents the first opportunity to reflect this in our methods of testing for pyrogenicity. The first interleukins were cloned in 1984, leading to an understanding of the mechanism of pyrogenicity: When an "exogenous pyrogen" enters the bloodstream, cells of the immune system produce "endogenous pyrogens" (interleukins) that signal the brain to generate a fever. The first human

blood-based *in vitro* pyrogenicity tests were developed over a decade ago (Hartung & Wendel *ALTEX* 12, 70-75; 1995), based on measuring the production of interleukins in response to the test substance. Such methods are physiologically and mechanistically relevant and thus are capable of detecting all classes of human pyrogens (though there are a few limitations, such as testing for contamination of drugs that interact with immune cells, however this limitation also applies to the rabbit test).

Human biology-based pyrogenicity tests have since undergone extensive development and evaluation. The five tests proposed for consideration vary in their use of human whole blood (fresh or cryopreserved), cells isolated from blood, or immune cell lines, and in the interleukin response they measure, but otherwise work on the same principle. It will be up to the panel to decide whether all of these test methods accurately model the pyrogenic response with the necessary accuracy and sensitivity, and whether it varies by application.

The proposed human biology-based tests have almost every advantage over the existing animal-based tests: They are more biologically relevant, more reproducible, and more broadly applicable than the animal-based alternatives. They are speedier, more cost-effective, less laborious, and more humane. They are very sensitive; as mentioned above, the whole blood IL-1 test has been shown to have a sensitivity of 10pg LPS/ml, far below the human fever threshold. (As previously discussed, the rabbit test is far less sensitive and consistent, and neither the rabbit or LAL tests have ever been formally validated to demonstrate either intra- and inter-laboratory reproducibility, much less their relevance to human beings. Thus, when the expert panel considers the proposed novel methods, it is especially important to avoid the common pitfall of using the animal data as the “gold standard” in assessing false positive and negative rates.)

In conclusion, the submitted BRDs represent an ideal opportunity to conduct an expeditious review of well-validated non-animal methods and fully replace outdated animal tests with modern, improved alternatives as per ICCVAM’s mandate. ICCVAM’s endorsement will be key in encouraging US government agencies and industry to develop the necessary confidence in these innovative methods. Led by the FDA, Agencies should require these tests as the new standards in place of the animal tests, for which there will be no adequate rationale for continued use. The rabbit test in particular should be deleted from pharmacopeias and regulatory guidance and not accepted by Agencies once the new tests are validated.

With all this in mind, we strongly urge ICCVAM to move ahead quickly to convene a panel of experts who can make the necessary scientific judgments regarding the proposed tests with a view towards a speedy affirmation of their respective values in assessing pyrogenic potential. Consumer safety, scientific rigor, and animal welfare concerns will all be best served by promoting the use of these accurate, sensitive, and humane tests.

Thank you for your attention and responsiveness to these comments.

Sincerely,

/s/

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/s/

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