Fever in the Test Tube – Towards a Human(e) Pyrogen Test

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Summary

The human whole blood IL-1 test exploits the reaction of monocytes/macrophages for the detection of pyrogens: human whole blood taken from healthy volunteers is incubated in the presence of the test sample in any form, be it a solution, a powder or even solid material. Pyrogenic contaminations initiate the release of the "endogenous pyrogen" Interleukin-1ß determined by ELISA after incubation. In order to understand any differences between the pyrogenic activity in this test and the existing live rabbit test (species differences versus aberrant response of the particular blood sample), the rabbit whole blood test was developed. This approach could also help to avoid the use of putatively infectious human blood for pyrogen testing in vitro.

Keywords: whole human blood test, pyrogen in vitro test, rabbit whole blood test, IL-1ß

Background Information

Pyrogen testing – *in vivo* and *in vitro* test methods

Pyrogens are a chemically heterogeneous group of fever-inducing substances derived primarily from gram-negative and gram-positive bacteria but also from viruses and fungi. They provoke an immune response by producing endogeneous pyrogens such as prostaglandins and the proinflammatory cytokines interleukin-1, interleukin-6 and tumour necrosis factor-α. Pyrogen testing of any pharmaceutical product for parenteral application is therefore imperative. Different methods using different protocols are in use.

In vivo Rabbit Test: The animals are injected i.v. with the drug and monitored for any reaction in the form of fever. This test is currently legally required by health authorities. The test, however, is subject to inherent problems, since the sensitivity of different species towards endotoxins varies by a factor of up to 10,000. Pyrogen testing currently requires about 200,000 rabbits each year in Europe. After a recuperation period of 2-3 weeks, the animals can be used again to test a new drug, providing that the test substances cause no permanent changes in the immune system of the rabbits.

Limulus Amoebocyte Lysate Test (LAL): This test measures the coagulation of the amoebocytes of the horseshoe crab, initiated by cell wall components (LPS) of gram-negative bacteria with a molecular weight of > 8000 daltons. The test cannot detect smaller LPS nor the LPS equivalents of gram-positive bacteria or fungi. Furthermore, the test cannot distinguish between the different types of endotoxins from gram-negative bacteria, which can vary in their fever-inducing potential in the mammal by a factor of 10,000 (Fennrich et al., 1999).

Human whole blood test (WBT): This test, using an ELISA, was developed in 1996 by Hartung and Wendel (Hartung and Wendel, 1996) and internationally validated by ECVAM (Hoffmann et al., 2005). It measures cytokine production, in this case IL-1ß, by human monocytes following a challenge with pyrogens. It is less expensive and more sensitive than the rabbit test and has the additional advantage of being able to examine the reaction strength directly in human material. Unlike the LAL, this test can detect not only endotoxins, but also lipoteichoic acids, fungi, and superantigens such as SEB (enterotoxin of Staphylococcus aureus).

Most recently, additional tests using interleukin-6 release as endpoint and peripheral blood mononuclear cells (PBMC) or the monocytoid cell line MONO MAC 6 (MM6) as a source for human monocytes were established. All these tests were scientifically validated (Hoffmann et al., 2005) and the validity approved by the ECVAM Scientific Advisory Committee.

Pyrogens induce the release of pyrogenic cytokines

The guaranteed absence of pyrogens is a critical safety precaution for all drugs administered parenterally, since these contaminants can pose a life-threatening risk of shock to the patient.

Contact with minute concentrations of endotoxia, the best known pyrogen – as low as 5 IU/ kg, i.e. 500 pg/kg bodyweight – cause multiple reactions in the patient: human monocytes release several cytokines, the most important being IL-1β, IL-6 and TNFα. The release of these cytokines can cause chills, rigors and hypotension. Furthermore, platelets can aggregate and the coagulation system become activated, resulting in dissemi-

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nated intravascular coagulation and organ hypoxaemia, multiple organ failure and death by shock.

Human whole blood test (WBT) can replace animal tests

Several tests are currently available to detect pyrogenic agents. Of these, a commercially available test using human whole blood (IPT) can detect a wide variety of pyrogens and is suitable for a broad range of applications (Fennrich et al., 1999; Hartung and Wendel, 1996; Jahnke et al., 2000).

Rabbit whole blood test: Bridging the gap between animal tests and WBT

Unfortunately, the response to pyrogens in the WTB can be affected by the donor of the blood sample. In order to establish the validity of the WBT for the pharmacopoeia, it is important to be able to understand any differences between the pyrogenic activity in the human whole blood test and the existing live rabbit test and to be able to attribute these differences to species differences or to an aberrant response of the particular blood sample (Fig. 1). As a link between the existing live test in rabbits and the WBT, the rabbit whole blood test was thus developed. This test uses the same species as the established *in vivo* test, but the same material and endpoint as the new *in vitro* test with human blood.

Several steps were necessary to establish the rabbit whole blood test: i) Production of recombinant rabbit IL-1β in *E. coli* (positive control for the ELISA, substance to immunise the animals in step two), ii) Immunisation of a sheep with the IL-1β antigen, iii) Immunisation of mice with IL-1β and production of monoclonal antibodies, iv) Establishment of a sandwich ELISA (Enzyme Linked Immunosorbent Assay) with the antibodies and the antigen.

These steps were successfully completed: recombinant IL-1β was produced in a reliable quality and sufficient quantity. Monoclonal (mice) and polyclonal (sheep) antibodies against rabbit IL-1β were isolated. The ELISA assay allows the quantitative determination of the endogenous rabbit fever signal IL-1β (Fig. 1).

Response to a pyrogen

The *in vitro* blood test allows the pyrogenic activity of various drugs and agents to be tested (Fig. 2), e.g. pentaglobin. Pentaglobin is a clinical human immunoglobulin preparation which is painful to the animal when administered intravenously in the live rabbit test. The rabbit whole blood test takes only four hours to perform and requires only 100 μl of blood per sample. Currently (in contrast to the human WBT), fresh blood has to be used; however, 7 ml of blood (i.e. enough for 70 samples) can be collected from one rabbit without any harm.

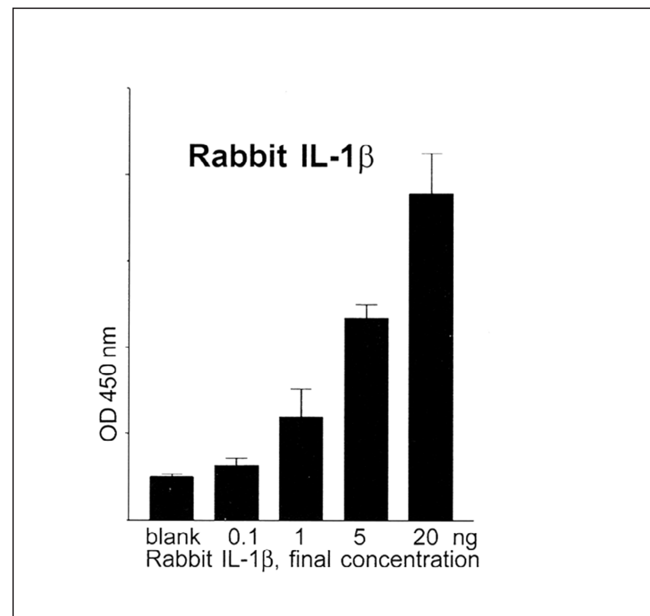

Fig. 1: Quantitative measurements of rabbit IL-ß with an ELISA

Tab. 1: Comparison of three pyrogen tests

		Test:	**Rabbit**	**LAL**	**WBT**
Pyrogens	Bacteria gram-negative		+	+	+
	Bacteria gram-positive		+	-	+
	Fungi		+	-	+
Applications	**Biologicals**	+		-	+
	Pharmaceuticals	+		+	+
	Medical Devices	-		+	+
	Air quality	-		(+)	+
	Blood components	-		-	+

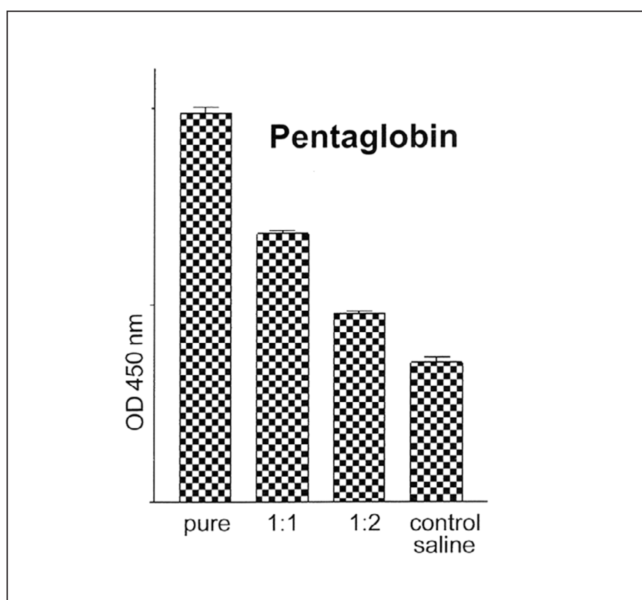

Fig. 2: TNFα release after incubation with a batch of pentaglobin (pure = final concentration: 10%) and serial dilutions

A promising approach

The whole blood test in rabbits can help to explain false-positive and false-negative results when comparing the WBT with pyrogen test results in live rabbits. Furthermore, pyrogen testing in animal blood makes it possible to examine species differences and test veterinary drugs in the target species (Schindler et al., 2002). In the future, this approach could also help to avoid the use of putatively infectious human blood for pyrogen testing *in vitro*.

References

Fennrich, S., Fischer, M., Hartung, T. et al. (1999). Detection of Endotoxins and other pyrogens using human whole blood. In F. Brown, C. Hendriksen, D. Sesardic (eds): *Alternatives to animals in the development and control of biological products for human and veterinary use*. Dev. Biol. Stand. (Vol 101, 131-139). Basel: Karger.

Fennrich, S., Wendel, A. and Hartung, T. (1999). New applications of the Human Whole Blood Pyrogen Assay (PyroCheck). *ALTEX 16*, 146-149.

Hartung, T. and Wendel, A. (1996). Detection of Pyrogens using human whole blood. *In vitro Toxicology 9*, 353-359.

Hartung, T., Crameri, R. and Wendel, A. (1998). Entwicklung eines Pyrogentests mit Kaninchenblut. *ALTEX 15*, 17-18.

Hoffmann, S., Peterbauer, A., Schindler, S. et al. (2005). International validation of novel pyrogen tests based on the human fever reaction. *J. Immunol. Meth. 298,* 161-173.

Jahnke, M., Weigand, M. and Sonntag, H.-G. (2000). Comparative testing for pyrogens in parenteral drugs using the human whole blood pyrogen test, the rabbit in vivo pyrogen test and the LAL test. *European Journal of Parenteral Sciences 5(2)*, 39-44.

Schindler, S., Bristow, A., Carmell, T. et al. (2002). Comparison of the reactivity of human and rabbit blood towards pyrogenic stimuli. *Altex 20*, 59-63.

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