Comparison of the Reactivity of Human and Rabbit Blood towards Pyrogenic Stimuli

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Summary
A comparison between humans and rabbits was performed based on stimulation of whole blood with well-known pyrogens from Gram-negative and Gram-positive bacteria, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA), respectively. The reactivity was measured as release of IL-1ß and IL-8 by ELISA. The reactivity of the two species towards LPS was found to be similar, whereas their reactivity towards LTA differed considerably. Differences between the levels of IL-1ß and IL-8 release were observed in both species. This finding suggests that the In vitro Pyrogen Test (IPT) which uses human blood to detect contaminations, e.g. of injectable drugs, might predict the human reaction to the contamination better than the "gold standard" rabbit pyrogen test.

Zusammenfassung: Vergleich der Reaktionsfähigkeit von Kaninchen- und Human-Blut auf Pyrogene
Es wurden wohlbekannte Pyrogene wie Lipopolysaccharide (LPS) aus Gram-negativen und Lipoteichonsäure (LTA) aus Gram-positiven Bakterien eingesetzt, um einen Vergleich zwischen Mensch und Kaninchen in analogen Vollblutmodellen vorzunehmen. Die Reaktivität wurde anhand der Freisetzung der Zytokine IL-1ß und IL-8 mittels ELISA beurteilt. Die Reaktivität der beiden Spezies gegenüber LPS war generell vergleichbar, während sich die gegenüber LTA beträchtlich unterschied. Ferner wurden Differenzen im Ausmass der Freisetzung von IL-1ß und IL-8 bei beiden Spezies beobachtet. Dieser Befund könnte bedeuten, dass der In vitro Pyrogen Test (IPT), bei dem humanes Blut eingesetzt wird, um Kontaminationen, z.B. von parenteralen Medikamenten, zu detektieren, verlässlicher die menschliche Reaktion auf Kontaminationen vorhersagt als der „Gold-Standard" Kaninchen Pyrogentest.

Keywords: comparison, human, rabbit, pyrogen, cytokine

1 Introduction

Pyrogens are fever-inducing substances derived for example from Gram-positive (lipoteichoic acid, LTA) or Gram-negative (lipopolysaccharide, LPS) bacteria. The contact of blood monocytes with pyrogens induces cytokine release, which in turn induces fever, hypotension and even lethal shock in the organism (Kluger, 1971). This may occur in sepsis or through the application of intravenously administered drugs that are contaminated with components of bacteria. The testing of such drugs for pyrogens has, therefore, been regarded as crucial since the middle of the last century. The *in vivo* rabbit pyrogen test, introduced into the United States Pharmacopoeia in 1942 is considered the gold standard for this safety measure. Briefly, after the injection of the substance intravenously, the individual changes of body temperature from pre-injection (baseline) values are measured and the injected substance is classified as pyrogenic or non-pyrogenic by calculating the sum of the rises in body temperature of usually three rabbits. The rabbit pyrogen test has certain ethical and practical drawbacks, e.g. the use of animals and the aspect of cost.

The Limulus Amoebocyte Lysate test (LAL) was established as an *in vitro* alternative to the *in vivo* rabbit pyrogen test. The LAL assay, however, fails to detect non-endotoxin pyrogens such as LTA, i.e. an immunostimulatory molecule from Gram-positive bacteria (Morath et al., 2001). Since contaminations with components other than endotoxin (one component of the cell wall of Gram-negative bacteria) are not unlikely or less dangerous, the inability to detect them is a major limitation of the LAL for the replacement of the rabbit test. Moreover, many drugs, in particular biologicals, cause interference with the LAL assay components and must therefore still always be measured *in vivo* in the rabbit pyrogen test. Importantly, the LAL also yields no information about the pyrogenic potency of a given endotoxin in the human being. The same might apply in principle to the rabbit (Fennrich et al., 1998).

For the above reasons, a new *in vitro* pyrogen test based on the human fever reaction modelled in human whole blood has been developed and validated over

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the past few years and hopes to replace the *in vivo* rabbit test (Hartung and Wendel, 1996; Schindler et al., 2002). The aim of the study reported here was to compare, *in vitro*, the sensitivity of humans and rabbits to various test substances and to correlate the *in vivo* findings in the rabbit with the *in vitro* findings in the novel pyrogen test. For this purpose, the 3R Research Foundation Switzerland sponsored the project of establishing a rabbit IL-1ß ELISA in order to allow the identification of species differences in analogous human and rabbit whole blood tests.

IL-1ß is one of the pro-inflammatory cytokines that are induced following the contact of the monocytes with pyrogens; the proinflammatory cytokines change the set-point for the thermoregulation in the brain and cause a fever reaction in the organism. Furthermore, they induce the production of acute phase proteins in the liver.

IL-8 is a chemokine that is produced by monocytes as well, but serves a different purpose; i.e. to attract other white blood cells to the site of infection by forming a concentration gradient and helping the successful extravasation of the white blood cells.

A number of novel *in vitro* pyrogen tests currently validated in a European collaborative study (Hartung, 2002) have proven to overcome the difficulties of the rabbit pyrogen test as well as the limitations of the LAL assay (final report to be submitted). These tests use either human whole blood or human blood cells/cell lines and therefore have the advantage of testing in the relevant species.

2 Animals, volunteers, materials and methods

The rabbits used were 8 male Chinchilla cross-breeds obtained from Charles River. The blood was drawn by venipuncture of the *Vena auricularis* into heparinised tubes purchased from Sarstedt (15 IU Li-Heparin/ml blood). The whole blood stimulation was performed within 2-3 hours of drawing the blood. The methodology of the whole blood incubation is described in detail elsewhere (Fennrich et al., 1998).

Briefly, heparinised whole blood from a healthy human donor or rabbit was diluted 12-fold in physiological saline and incubated together with the respective stimulus for 10-24 hours at 37°C. After resuspension and centrifugation, the cytokines in the supernatants were

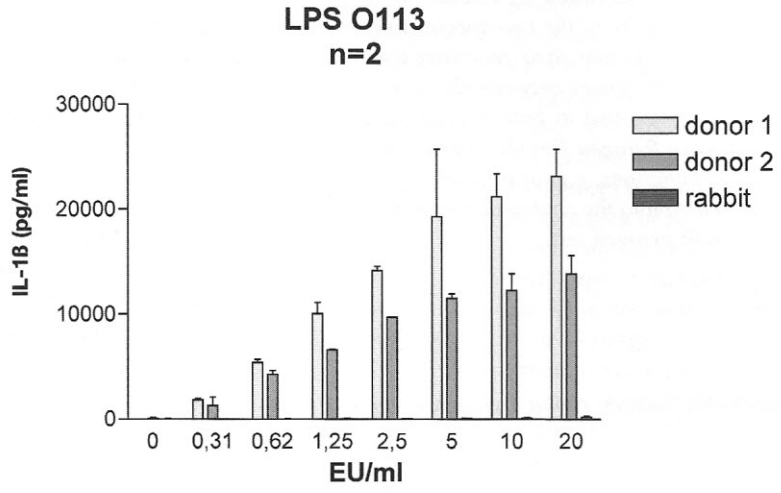

Fig. 1: IL-1ß; comparison of blood from two human donors and one rabbit concerning reactivity towards the *E. coli* LPS O113; n=2 (n: number of replicates/error bars in graphs 1 and 2 are min-max)

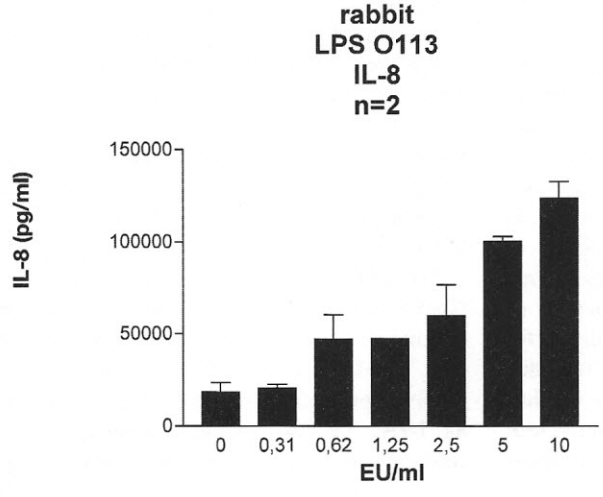

Fig. 2: Measurement of rabbit IL-8 from the same experiment; n=2

measured by ELISA. The standard procedure was modified with regard to the volumes employed due to the limited amount of blood that could be obtained in one session without harming the rabbits; the total volumes of the incubations were reduced from 1.2 ml to 0.6 ml in the human as well as the rabbit whole blood incubations.

The LPS preparation from *Serratia marcescens*, a Gram-negative bacterium, was a phenol extraction from Sigma; the LPS from *E.coli* O113:H10 was a preparation from the NIBSC. The LTA from *B. subtilis* was an in-house preparation (Morath et al., 2001) included in the IPT (*In vitro* Pyrogen Test) distributed by Charles River as a Gram-positive control. The *E. coli* LPS O111 serves as the Gram-negative stimulus in the above-mentioned IPT. Briefly, the pyrogenic threshold was determined by diluting the pyrogens until no significant cytokine response could be measured. This threshold, i.e. the first concentration inducing the respective cytokine, varies from pyrogen to pyrogen, e.g. by a factor 10,000 between LPS from different bacterial species. Due to this fact, the pyrogenic potency of a substance is determined using the WHO reference standard LPS O113; for this LPS, 100 pg/ml equals 1 EU/ml (EU = endotoxin unit). The potency of other pyrogens was compared with that of the LPS O113 and expressed in EU/ml.

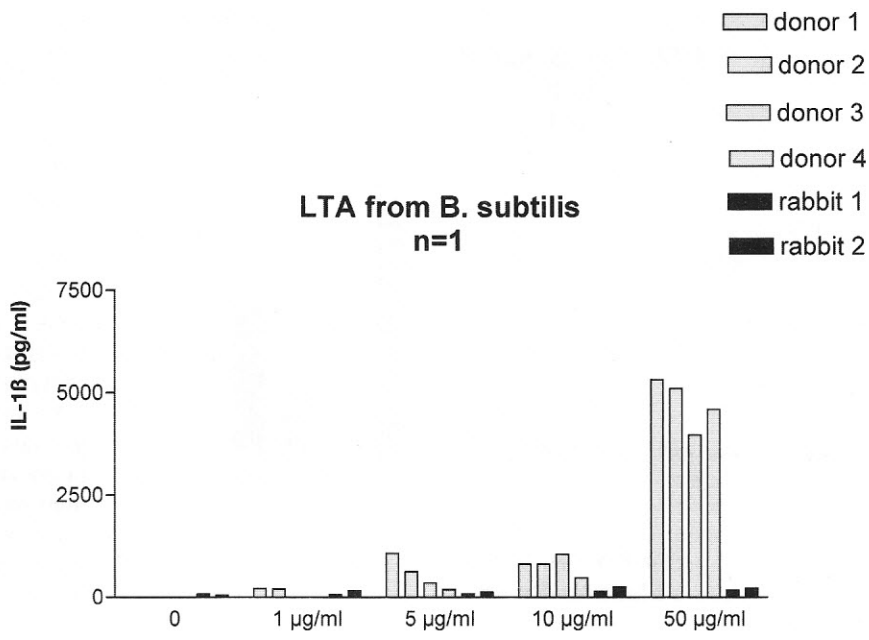

Fig. 3: IL-1ß response of 4 human donors and 2 rabbits to LTA from *B. subtilis*; n=1

The reagents for the rabbit IL-1ß ELISA were prepared at the NIBSC. Rabbit IL-1ß was expressed and purified in recombinant *E. coli*. Briefly, the coding sequence of the mature rabbit IL-1ß protein was amplified by PCR from activated rabbit monocytes and cloned into the vector pET24a (Novagen; USA), between the Nde-I and Xho-I sites. Expression of IL-1ß as a soluble, cytoplasmic protein was induced by IPTG and the protein was purified by ammonium sulphate precipitation (50% saturated), gel filtration (Sephadex G-75, 0.05 M ammonium acetate), lyophilisation and ion-exchange chromatography (Mono-Q, 0.025M Tris-HCl pH 8.5, NaCl gradients). Rabbit IL-1ß was recovered as a pure (> 98% by SDS PAGE) protein, containing < 10 ppm endotoxin. Antisera to rabbit IL-1ß were raised in sheep using standard immunisation protocols. The sheep anti-rabbit IL-1ß serum was a gift from Stephen Poole, Division of Endocrinology, NIBSC, and was raised against recDNA-derived protein kindly provided by Alan Shaw of GSK.

The rabbit IL-8 ELISA was a commercial ELISA kit provided by PharMingen (USA); the recommended procedure was modified with regard to the antibody concentrations and the buffers used.

The reagents for the human IL-1ß ELISA were purchased from Endogen (USA) as well as the reagents for the human IL-8 ELISA system. ELISA plates were MaxiSorp 96 well flatbottom plates provided by Nalge Nunc International (Denmark). Binding of biotinylated antibody was quantified using streptavidin-peroxidase (Biosource, Camarillo, CA, USA) and the substrate TMB (3,3',5,5'-tetramethylbenzidine, Sigma).

3 Results

The rabbit IL-1ß and IL-8 ELISAs had sensitivities of 7 and 4 pg/ml, respectively. The reactivity of the rabbit blood to LPS was comparable to that of the human blood. Interesting exceptions were an occasional complete lack of reactivity of the rabbit blood towards the reference standard O113. Although only some animals' blood samples showed this lack of response, it was highly significant when it did occur (Fig. 1).

The rabbit blood from the same experiment did nevertheless react to *E. coli* LPS O113 by releasing IL-8 (Fig. 2).

When IL-8 was measured, the reactivity of rabbit blood was comparable to that of human blood regarding both LPS and LTA response. The reactivity towards LTA differed significantly between rabbit and human blood with a reduced or completely lacking release of IL-1ß by the rabbit blood in response to this stimulus. This indicates that LTA is

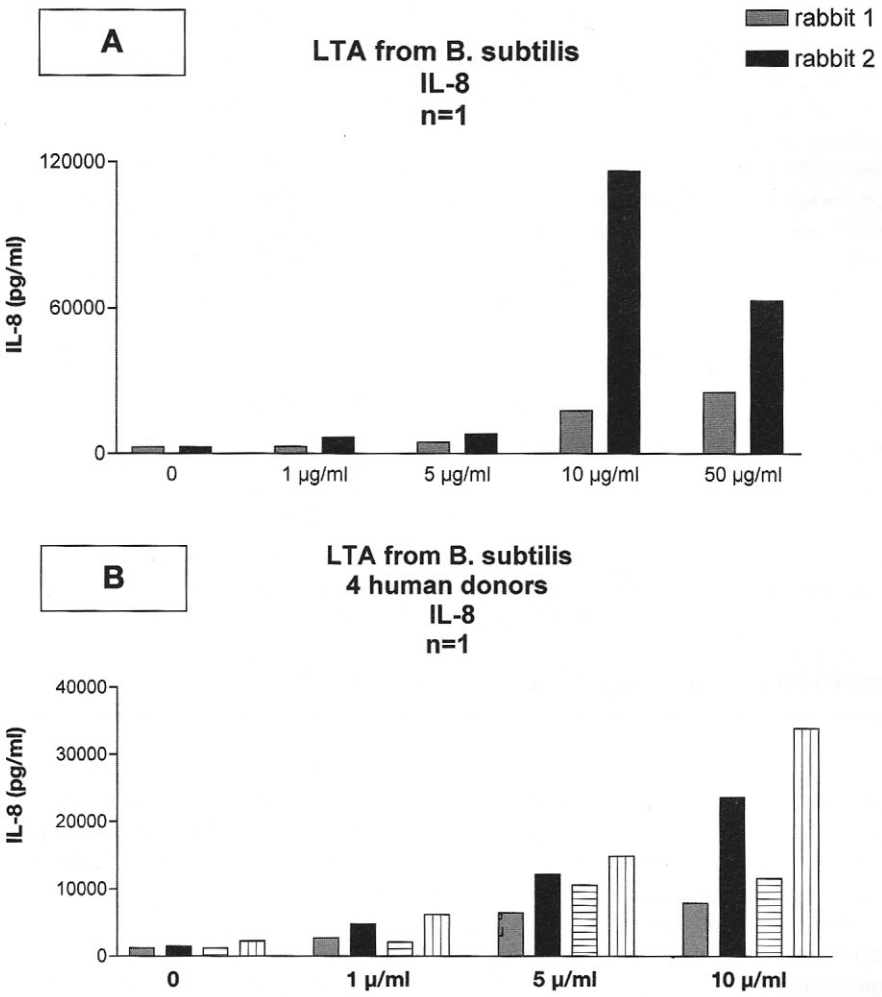

Fig. 4: IL-8 response of rabbit (A) and human blood (B) to LTA from *B. subtilis*; n=1

recognised by the rabbit immune system but that the response is selective.

The following example shows a stimulation experiment employing LTA from the Gram-positive spore-former *Bacillus subtilis* – this material is provided in the commercial IPT Kit as a Gram-positive control (Fig. 3, 4).

The threshold for a response to the *Bacillus subtilis* LTA is considerably higher in rabbit blood than in human blood. The same however again does not apply to the IL-8 readout (Fig. 4a, 4b).

For this measurement, the reactivity of the rabbit and the human blood was identical. Although the threshold reactions are difficult to judge since only one value per animal could be run for each concentration, significant IL-8 release was observed at 5 µg/ml in all cases (Tab. 1).

4 Discussion

In the last three years, a European consortium for *in vitro* pyrogen testing has devoted itself to the refinement and evaluation of different cell-based test systems all over Europe (Schindler et al., 2002). The project reported here draws an *in vitro* comparison of the merits and drawbacks of a rabbit and a human pyrogen test based on whole blood. The results on non-endotoxin pyrogens and the huge species differences shed a critical light on the "gold standard" rabbit

Tab. 1: Summary of the results obtained with different pyrogens

LPS/LTA	Threshold rabbit IL-1	Threshold human IL-1	Threshold rabbit IL-8	Threshold human Il-8
Serratia marcescens	20-50 pg/ml	10-20 pg/ml	n.d.	10-20 pg/ml
O113	50-100 pg/ml (4 rabbits), no reaction (2)	12.5-25 pg/ml	12.5-25 pg/ml	12,5-25 pg/ml
O111	0,125-0,25 EU/ml	0,0625-0,125 EU/ml	0,25 EU/ml	0,0625 EU/ml
LTA from *Staphylococcus aureus*	> 100 µg/ml	5 µg/ml	10 µg/ml	1-5 µg/ml
LTA from *Bacillus subtilis*	> 100 µg/ml	5 µg/ml	5 µg/ml	5 µg/ml
Salmonella abortus equi	100-200 pg/ml	50-100 pg/ml	100-200 pg/ml	50-100 pg/ml

(n.d. = not done)

test and its ability to detect non-endotoxin pyrogens if the *in vitro* findings can be translated to the *in vivo* situation. The difference in the response to the O113 LPS should not be overinterpreted, although it was highly significant when it occurred, because the number of experiments is still insufficient and this question has to be studied further. Another difficulty is the high variance in the rabbit blood results with a high percentage of outliers; this is probably due to the long and difficult procedure of drawing blood, which results in minor contaminations which may cause a disturbance, especially in the range of low stimulus concentrations. Therefore, in general, a high number of replicates must be done to be able to exclude outliers. For this reason, the threshold cannot be definitely determined in many experiments. The same problems, however, do not apply to the human whole blood incubations. The variance of the rabbit blood in between the experiments was a problem which can only be solved by a larger number of experiments and a systematic approach investigating the reactivity of the blood of the individual rabbits on different days.

The variable reactivity towards the LPS O113 is difficult to explain, since the reactivity and the thresholds of human blood to LPS in general are highly conserved. Another interesting point is the difference between IL-1ß and IL-8 response; IL-8 proved to be a highly sensitive parameter with no false-negative results in the rabbit blood, whereas the IL-8 levels in human blood were comparable to those of IL-1ß. It will be important to clarify what causes this difference as well as the role and significance of these two cytokines in the rabbit.

The significance of the high levels of IL-8 (up to 120 ng/ml) produced by the rabbit blood is unclear. It will be interesting to see whether rabbits show similarly high levels of IL-8 *in vivo*. One problem of the rabbit IL-8 ELISA assay was a high blank due to a cross-reaction of the antibodies with an unidentified component in the blood, as could be determined by diluting unstimulated rabbit serum in saline and measuring it in the ELISA (data not shown). A certain percentage of the high IL-8 production measured can be explained by this phenomenon.

A future project will be the measurement of the cytokines IL-6 and tumor necrosis factor (TNF), two other proinflammatory cytokines, in order to determine whether the differences in the reactivity of human and rabbit blood are also reflected in these cytokines.

The *in vitro* rabbit test will be adapted to other non-endotoxin pyrogens such as phytohemagglutinin, and components of fungi or yeast in order to compare the rabbit and the human response to these substances. In light of the successes of the human blood *in vitro* pyrogen test and considering the species differences demonstrated in this study, an interesting prospect would be to establish similar tests for other animal species in order to improve the safety control and quality assessment of intravenous drugs in veterinary medicine. Another possibility might be the testing of veterinary drugs that cannot be tested *in vivo*, such as immunoglobulins, blood products, etc., for possible immunostimulatory contaminations.

A further avenue to be explored is the testing of drugs with artificial or natural contaminations in the rabbit *in vivo* and *in vitro*. Work on critical substances such as albumins has already been started *in vivo* (Spreitzer et al., 2002). In conclusion, these pilot studies in rabbit blood demonstrate that species-specific pyrogen tests offer the possibility to compare different species and to extrapolate to the *in vivo* situation.

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