

5. Discussion

Part 1: Comparison of the in vivo fever reaction of the rabbit with the in vitro cytokine production measured by ELISA

It could be asserted that the in vitro rabbit pyrogen test based on an incubation of rabbit whole blood and measuring cytokine production was indeed a suitable replacement for the in vivo test, where the pyrogen is injected intravenously into the rabbit and the fever reaction is monitored. Taking a blood volume of 40-50 ml/kg bodyweight of an adult rabbit (Mott, 1967), an LPS dose of the WHO endotoxin standard of 40 EU/kg corresponded exactly to the first LPS dose of the same standard in vitro of 0.75-1 EU/ml that induced a significant cytokine response (Fig. 1 and 2), indicating that the in vitro pyrogen test is a relevant tool for mimicking the in vivo reaction.

In the in vivo experiment, 3 cytokines were measured by drawing blood from the animals at different time points at and after intravenous pyrogenic challenge. Unlike in the in vitro assay, no IL-1 β could be detected throughout the duration of the experiment, and TNF- α was measurable only at 30 minutes after endotoxin injection and was not detectable half an hour later. This is in concordance with the findings of Cannon et al., who did not detect IL-1 β prior to the onset of fever (Cannon et al., 1990), to those of Engel et al., 1994 and of van Zee et al. 1995, who all failed to detect circulating IL-1 β levels in human subjects in naturally occurring sepsis or after endotoxin injection and with those of Bristow et al., 1991 and Cooper et al., 1991, who did not find IL-1 β in the blood of febrile rats and mice. Still, the fact that IL-1 β is a potent endogenous pyrogen capable of inducing fever was asserted by Dinarello et al., who injected different concentrations of recombinant, endotoxin-free human IL-1 β intravenously into rabbits and found that as little as 8 ng/kg can induce a fever reaction of 0.6 °C (Dinarello et al., 1991). The pyrogenic properties were also shown by other authors, e.g. Murakami et al. in 1990 and by Morimoto et al., 1990, although it must be taken into account that they used comparatively high concentrations of intravenously applied cytokine (0.2 and 0.5 μ g/kg, respectively), which could impede the latter's results by producing pathologically high plasma levels.

The appearance of TNF- α 30 minutes after injection, immediately before the body temperature of the rabbits began to rise might indicate a causative role in thermogenesis. Dinarello et al., showed in 1986, that intravenous injection 1 μ g/kg of human endotoxin-

free recombinant TNF- α induced fever in rabbits. This concentration of 1 $\mu\text{g}/\text{kg}$ of TNF- α is also very high when compared to the blood level of $< 50 \text{ pg}/\text{ml}$ in vivo found at the NIBSC. It is possible, though, that the human recombinant cytokine is not as potent in the rabbit as the natural cytokine would be. In general, the doses of TNF- α necessary to evoke a significant fever reaction were very high (Nakamura et al., 1988, Kettelhut et al., 1988). On the other hand, injection of 4 ng/kg of LPS into human subjects resulted in fever and in elevated plasma levels of TNF- α (Michie et al., 1988, Suffredini et al., 1989). In contrast, Engel et al were not capable of detecting circulating TNF- α in endotoxemic humans, maybe due to the short persistence of this cytokine in the plasma (Engel et al., 1994).

The in vivo data generated by NIBSC indicate that low blood levels (in the case of IL-6 150 pg/ml) occur in a severe fever reaction of $> 0.6^\circ\text{C}$. Dinarello et al. were able in 1991 to evoke fever in rabbits by injection of 200 ng/kg of recombinant human IL-6. The causative role of IL-6 in the pathogenesis of fever however must remain doubtful, since the peak IL-6 level occurs only after the onset of the temperature rise (Fig. 1). Good correlations between the IL-6 plasma levels and fever were also found by Nijsten et al. in 1987, and elevated plasma levels of IL-6 and IL-8 by Engel et al., 1994. Since TNF- α can be measured only for a very short time and IL-1 β is generally undetectable, it must be taken into consideration that messengers this potent are probably quickly bound to their corresponding receptors, e.g. to the Interleukin-1 receptor antagonist (Luheshi et al., 1996) or other binding proteins, or internalized, e.g. by binding to/crossing the blood-brain barrier (Banks et al., 1991, Banks et al., 1994), making them inaccessible to the antibodies of the ELISA system, or otherwise quickly eliminated.

The role of the respective cytokines in the generation of a fever reaction could not be determined in this set of experiments. It could be speculated, though, that IL-1 β is rapidly eliminated from the blood stream because it is a very potent fever inducer, and enters the anterior hypothalamus, inducing local IL-6 production and fever (Klir et al., 1994, Chai et al., 1996). Nevertheless, the in vitro results, where all 4 readouts could be measured showed that all are useful for determining pyrogenic stimulation, although the TNF- α endpoint was less sensitive than the others, indicating that even if a cytokine is not the cause of the fever itself it can still be a useful parameter in pyrogen detection.

The supernatants of two rabbits could be remeasured in the TNF- α and IL-6 ELISA once they were established. Interestingly, one rabbit failed to respond altogether with an IL-1 β response with an at the same time strongly (4-5 times more than the other 6 rabbits)

elevated IL-8 response and the rest of the measured endpoints showed a 5-10 times reduced sensitivity compared to the other rabbits. The high IL-8 production proves that the lack of IL-1 β is not due to a hyporesponsiveness of the monocytes of this particular animal, but must have other reasons. Why and how this phenomenon came about, could not be addressed in this study. This is not the first time that this occurred with this particular LPS, and it appears to be in line with in some in vivo findings of NIBSC where no fever reaction could be induced even with high doses of *E. coli* O113: H10 (Dr. Tammy Cartmell, NIBSC, personal communication). The same held true for the stimuli from Gram-positive bacteria (Schindler et al., 2003), especially from *B. subtilis*. The failure to react to the LPS from *E. coli* O113:H10 could be attributed to individual defects in the receptor-transmitter axis were it not for the perfectly normal reaction to other types of LPS in the very same experiment. Endotoxic reactivity in vivo does depend on the structure of lipid A (Rietschel et al., 1993), especially on the content of hydroxyacyl residues (Tanamoto et al., 1984). The LPS from *E. coli* O113: H10 is one of the most potent reagents employed in this study, maybe indicating that a structure specific for this endotoxin is not recognized by every animal. This might indicate that the human in vitro assay is even more reliable than the rabbit pyrogen test, since a substance that is pyrogenic for humans would maybe have yielded a false-negative response in vivo and in vitro.

The determination of the roles of the respective pyrogens in fever, sepsis and acute phase response has yet to be determined. The new highly purified recombinant rabbit cytokines might be helpful in elucidating these roles in vivo and in vitro. Another interesting new tool are specific gene-deficient mice, e.g. IL-1 β and IL-6 knock-out mice, as were recently used e.g. in a study of Kozak et al. LPS injection caused attenuated fever in IL-1 β KO mice, but not in IL-6 KO mice (Kozak et al., 1998). IL-1 β injection into deficient mice produced a fever reaction (Alheim et al., 1997). Another approach is the development of neutralizing antibodies which can be used to specifically block cytokine function in vitro or in the organism (e.g. IL-1 β receptor antagonist). Evidence is accumulating, though, that the cytokines and eventually the pyrogens themselves act upon their receptors at the blood side of a structure in the third ventricle (OVLT, organum vasculosum laminae terminalis) without crossing the blood-brain barrier (Blatteis et al., 1992), inducing the brain side to produce PGE₂. It is interesting to note that TNF- α and IL- β both can activate endothelial cells in vitro to synthesize and release PGE₂, (Rossi et al., 1985) which in turn, not being a messenger itself, induces a substance

that causes the change of the temperature set point in the hypothalamus. A candidate molecule is cAMP (Laburn et al., 1974, Rosendorff et al., 1976).

Part 2: Comparison of the in vitro reaction of humans and rabbits towards a variety of stimuli

When the WHO endotoxin standard used in part 1 was employed in the human whole blood test, all 4 readouts proved to be excellent and reliable parameters. The most uniform and sensitive response could be evoked with the IL-1 β endpoint. All donors responded at LPS concentrations of around 0.5 EU/ml. When in vivo rabbit experiments were done at the PEI, using the WHO standard as well, 50 % of altogether 171 rabbits responded even at a challenge of 5 EU/kg, applied in 10 ml/kg. (Hoffmann et al., in press). All rabbits responded at 20 EU/kg. From these data derived the limit concentration of 0.5 EU/ml as the threshold of pyrogenicity for parenterals in human medicine that was the basis for a validation study that established new cell-based pyrogen tests, amongst which is our human whole blood assay measuring IL-1 β (Hoffmann et al., 2005). This threshold is further supported by the study of van Zee, where humans injected intravenously with 20 EU/kg of WHO endotoxin standard developed moderate fevers (van Zee et al., 1995). Why the rabbits at the PEI responded more sensitively towards the endotoxin challenge is impossible to say, but could be due to various differences in age, sex, (Deeter et al., 1989), breed, handling, e.g. restraint measures (Grant, 1950, Sheagren et al., 1966), etc. It is well known that all these parameters can change and impede the reactivity of rabbits. Especially differences in pyrogen sensitivity of different breeds is a well-studied phenomenon (van Dijck et al., 1977).

Having shown the comparability of the readouts with the WHO endotoxin standard, the newly established in vitro rabbit pyrogen test and the in vitro human pyrogen test was tested with 6 Gram-negative and one Gram-positive stimulus. The procedure of the rabbit assay was identical to the already existing human whole blood protocol in order to ensure maximum comparability. First, two more types of *E. coli* LPS were used, one with a high pyrogenic activity and one with a lower one. Both species tested responded extremely sensitively to the former and much less to the latter, indicating that the recognition and inducing pathways of both species are extremely similar. Greisman et al. could show in

1969 that both humans and rabbits showed the same sensitivity towards endotoxin from *E. coli*. In his study, as little as 1 ng/kg of this endotoxin evoked a fever reaction in man and rabbit, although the exact type of LPS is not specified. This shows the usefulness of the concept of the endotoxin units (EU), where an amount of endotoxin is expressed as a measure of activity and not of weight. This way, pyrogenic stimuli employed by different groups become much more comparable, since the activity even of the same type of LPS can differ strongly depending on the purification methods, degree of purity, reconstitution, storage etc.

The two endotoxins from *Salmonella* were again one with a high cytokine-inducing capability and one that had proven to be less active in previous experiments. The more potent LPS from *Salmonella typhosa* was again very comparable in both species, which is again in concordance to the findings of Greisman et al., 1969, who found 1.0-1.4 ng/kg of this endotoxin to evoke a minimum febrile reaction. For the LPS from *Salmonella typhimurium* on the other hand, an approximately 4 fold reduced sensitivity for the rabbit could be seen. It is known that the doses of LPS from *Salmonella typhimurium* necessary to elicit a fever response in the rabbit are relatively high (e.g. Elin et al., 1976) but we could not find any data on the use of this LPS in humans. Since the in vitro assays worked very well by this time it was assumed that this finding represents a genuine difference in the sensitivity of humans and rabbits.

In order to test a wider variety of types of endotoxin, two more LPS were used that had proven to be very active in the human whole blood assay in previous exploratory experiments. Rabbits in general responded very well to the LPS from *Serratia marcescens*, as did the humans, but not as sensitive towards *Shigella flexneri*.

As a last step, a highly pure and endotoxin-free preparation of lipoteichoic acid (LTA) derived from the cell walls of the Gram-positive bacterium *Bacillus subtilis* was used. In former experiments, a lack of response of rabbits had been observed with the IL-1 β readout (Schindler et al., 2003). The rabbits seemed to be less sensitive towards this stimulus than the humans, but a total lack of IL-1 β production could not be reproduced in this set of experiments, which used rabbits different from before, indicating that a hyporesponsiveness might be an individual phenomenon.

The rabbits responded in general very sensitive to all types of LPS. Throughout, the IL-1 β endpoint was the most sensitive and reliable one, with the smallest variability. This

cytokine has for a long time been recognized as a key mediator and the most potent endogenous pyrogen when injected intravenously (Dinarello et al., 1991).

Notable differences in the sensitivities existed for the LPS from *S. typhimurium*, *Shigella* and the Gram-positive stimulus from *B. subtilis*, partly confirming earlier findings, in which rabbits showed no response to pyrogen derived from Gram-positive bacteria. A strong reaction towards all stimuli, especially the Gram-positive one, could be observed for IL-8. This result has already held true for the human assay (Standiford et al., 1994, von Aulock et al., 2003). Since no in vivo data were done in parallel, it is impossible to answer the question whether a fever reaction would have occurred in these rabbits at this time.

It could be shown that in general rabbits produce less cytokine/ml blood than humans. In order to interpret these findings correctly, it must be noted that the sensitivity of the rabbit IL-1 β ELISA was exactly the same as that of the human IL-1 β ELISA (6.7 pg/ml), whereas that of the rabbit IL-6 ELISA system was about tenfold less than the human IL-6 (around 60 pg/ml vs. 6 pg/ml) so small amounts of IL-6 might not have been recognized. The contrary was true for the rabbit IL-8 ELISA, which showed a certain basic stimulation even when only saline was added to the rabbit blood, but at the same time generating up to 1 μ g IL-8/ml rabbit blood. This was due to a slight cross-reaction of the antibodies to an unidentified component of the rabbit plasma, as could be shown with unstimulated commercial rabbit serum.

Still, in the light of maximum responses of up to 1000 ng/ml a certain level of about 6 ng/ml does not influence the findings and it did not seem to influence the sensitivity. The sensitivity of the IL-8 readout could have been even better had it not been for the extremely high variance that impaired later significance in the evaluation. Furthermore, the amount of IL-8 appeared to decrease in some cases after a certain level had been reached. This can be explained from a tendency of the monomeric IL-8 molecule to form dimers at a certain concentration (Burrows et al., 1994) and from the fact that it binds to erythrocytes (de Winter, 1997).

TNF- α was the least sensitive readout for both species. This might be due to the fact that only little amounts of TNF- α are produced by the monocytes and that the detection limits of the ELISAs are not good enough to detect these levels (ca. 20 pg/ml for the human and 20-60 pg/ml for the rabbit ELISA). On the other hand it is possible that the assay procedure is not optimal for this specific cytokine. Probably the assay can be improved by using e.g. cell culture medium instead of saline for the whole blood incubation.

The in some cases high “blank” seen in some rabbits is most probably due to the drawing of the blood. Since the multily needle set for the humans could not be used with the rabbits, a 21 Gauge needle had to be taken. For this purpose, the tube had to be screwed open, which of course does not allow a totally sterile and pyrogen-free environment. A small basal cytokine production, especially notable for the IL-8, is therefore regrettable, but not always completely avoidable.

The most comparable readout with the best ELISA systems (both with a detection limit of 6.7 pg/ml, the best control values and the best variance) was the IL-1 β ELISA. IL-6 often failed to detect low LPS concentrations. This could be due to the relatively high detection limit of 60 pg/ml.

It is notable that for many endotoxins, the interindividual differences tend to be higher in the rabbit than in the humans. This might be in concordance to the findings of Stitt et al. in 1985, who examined the fever reaction of 26 rabbits after the application of endogenous pyrogens, and found them to develop temperature rises that varied between 0.4 °C and 1.5 °C, and therefore was able to divide them into groups of small, intermediate and large responders. A large responder for the humans was found in one case for the LPS of *E. coli* O111: B4 (Fig. 12, 14, 16) where one donor produced 4-5 times more IL-6, IL-8 and TNF- α than the other four. Interestingly, this phenomenon did not occur with the IL-1 β readout. Otherwise, the human donors were very homogenous concerning their sensitivity and cytokine response. The experiments were done in parallel, whenever possible, the ELISAs and the blood incubation procedure were highly standardized, and the responses in between different experiments performed on different days were highly similar. Furthermore, all animals were of the same breed, age and sex. Therefore, it can be assumed that the rabbit indeed has a higher variability in sensitivity than the human being, although some of the differences might be due to the handling of the rabbit blood.

Part 3: Less potent Gram-negative stimuli in the rabbit IL-1 β - and the rabbit IL-8 ELISA.

In the light of the lack of reactivity of some rabbits towards LPS from *E. coli* O113 or LTA and the decreased one in the case of *Salmonella typhimurium*, it interested us whether comparable phenomena existed for other stimuli. So far, only very potent LPS

had been used, with the exception of perhaps *S. typhimurium*. Furthermore, it had been shown for the mammalian cell that for several endotoxins, the pyrogenicity can differ strongly depending on whether it is measured by the Limulus amoebocyte Lysate (LAL) test or in the rabbit. Ray et al. could show in 1991 that LPS from *Bordetella pertussis*, had a 1000 fold lower activity in a cell based assay employing human monocytes than the LAL. A similar phenomenon occurred when Wachtel et al. and Weary et al. compared the activity of *Pseudomonas aeruginosa* LPS in the LAL and in the rabbit pyrogen test (Wachtel et al., 1977 and Weary et al., 1982). When the endotoxins of *Bordetella pertussis* and *Pseudomonas aeruginosa* were tested in our lab, it turned out that the detection limit for the former was 10-100 pg/ml in the LAL and 10 ng/ml in the human whole blood assay and for the latter 1 ng/ml and 100 ng/ml, respectively.

Therefore, these two LPS were used, measuring only rabbit IL-1 β and IL-8. In all cases, the IL-8 response was ca. 10fold the IL-1 β , but the cytokine reaction was around the detection limits of the human whole blood assay and not the LAL.

This shows that sensitivities of both humans and rabbits are highly similar with endotoxins of all ranges of potency (10^{-12} – 10^{-6} g/ml). The LAL, in contrast, as an assay employing material from a nonvertebrate, cannot distinguish in a comparable way between these different types of LPS (Hartung et al., 2000). That proves that the biological relevance of a contamination can only be judged in the mammalian system. After all, the human whole blood assay as well as the rabbit whole blood system is a valuable tool for determining pyrogenic contaminations and predicting their capability for fever induction.

Part 4: Establishment of a WEHI assay measuring human and canine TNF- α production after stimulation of whole blood

A WEHI assay for the TNF- α measurement of canine supernatants was established and compared to the human response. The WEHI system proved to be a good tool for measuring human and canine cytokine production. The factors of the human ELISA and WEHI readouts were highly similar. The readouts of the ELISA system were considerably higher than those of the bioassay, which is due to the fact that the bioassay can only measure bioactive TNF- α , whereas the ELISA antibodies capture the total amount produced by the monocyte. The readouts of canine TNF- α bioassay were lower

than the human readouts, meaning either that the dog makes less TNF- α than the human or that less cytokine is detected in the WEHI due to an incomplete cross-reaction of the murine cells with the canine cytokine. Anyway, the WEHI for canine whole blood seems to be a tool that can be further explored and probably improved. No differences concerning the limits of detection could be detected when the canine and the human whole blood were compared with the LPS from *Salmonella abortus equi*, and in the further experiments the cells showed a reaction even at comparatively low doses of stimulus. The good correlation between the immunoassay and the bioassay is an excellent result; several authors have complained about lacking comparability between these two readouts (Duncombe et al., 1988, Fomsgaard et al., 1988, Petersen et al., 1988).