

1. Introduction

The measurement of pyrogens is an important safety measure for parenterally applied drugs. Pyrogens (greek pyros: fire) can cause fever, hypotension and shock with organ failure and ensuing death. Pyrogens can be the so-called endotoxins or lipopolysaccharides (LPS), cell wall components of Gram-negative bacteria such as *E. coli*, *Salmonella* or *Bordetella*, but also of the cell walls from Gram-positive bacteria, e.g. so-called lipoteichoic acids (LTA). Pyrogens have been measured with the so-called in vivo rabbit pyrogen test, where drugs are injected into at least three rabbits and a fever reaction is measured by a rectal probe, or by the LAL (Limulus amoebocyte lysate test) based on a coagulation reaction of the amoebocytes of *Limulus polyphemus* when brought into contact with endotoxins.

Recently, a novel way of pyrogen testing has been introduced, which employs the monocytes of human whole blood. They produce proinflammatory cytokines, e.g. Interleukin 1 β (IL-1 β) or TNF- α and chemokines when brought into contact with pyrogens. These can be measured by ELISA technique or, in the case of TNF- α , also by the WEHI bioassay employing murine fibrosaroid cells.

Pyrogen testing and complications during intravenous treatments have been a major issue in human medicine for the past 50 years. The new in vitro pyrogen test based on human whole blood has been validated recently and has proven to be a reliable and safe tool for a wide range of applications, not only drugs, but also medical devices and even quality testing of air in animal stables. An analogous development is principally possible for all species and several aspects of veterinary medicine. The testing of e.g. vaccines, antibiotics or other parenterally applied drugs could be an interesting and important topic if, for example, many animals are treated at once (pigs, cattle). Adverse reactions after treatment could possibly be prevented by a prior test, maybe even with animals from the stable in question. A further possibility is the measurement of the quality of stable air extrapolating to the animals themselves.

By employing rabbit blood, this study connected the historical in vivo pyrogen test in the rabbit with the new in vitro pyrogen test by establishing an in vitro rabbit test which runs analogous to the human whole blood test. Additionally, it was shown that pyrogen testing can be performed not only measuring IL-1 β , but with other cytokines as well and that for some species even a cell-based test can be done. The overall aim is to make species-specific pyrogen testing available for veterinary medicine. It had to be shown that specific assays can be established and that pyrogen measurements based on the whole blood

incubation method are possible, safe and easy to perform. In a series of experiments performed by members of the National Institute of Biological Standards and Controls (NIBSC), rabbits were challenged in vivo by injecting different doses of endotoxin, in this case the international WHO standard O113:10 derived from the Gram-negative bacterium *E. coli*. 100 pg/ml of this specific endotoxin has the biological activity of 1 Endotoxin Unit (EU)/ml. After injection, the rise in temperature was measured by a rectal probe and the blood cytokine production was measured (Fig. 1, data courtesy of NIBSC).

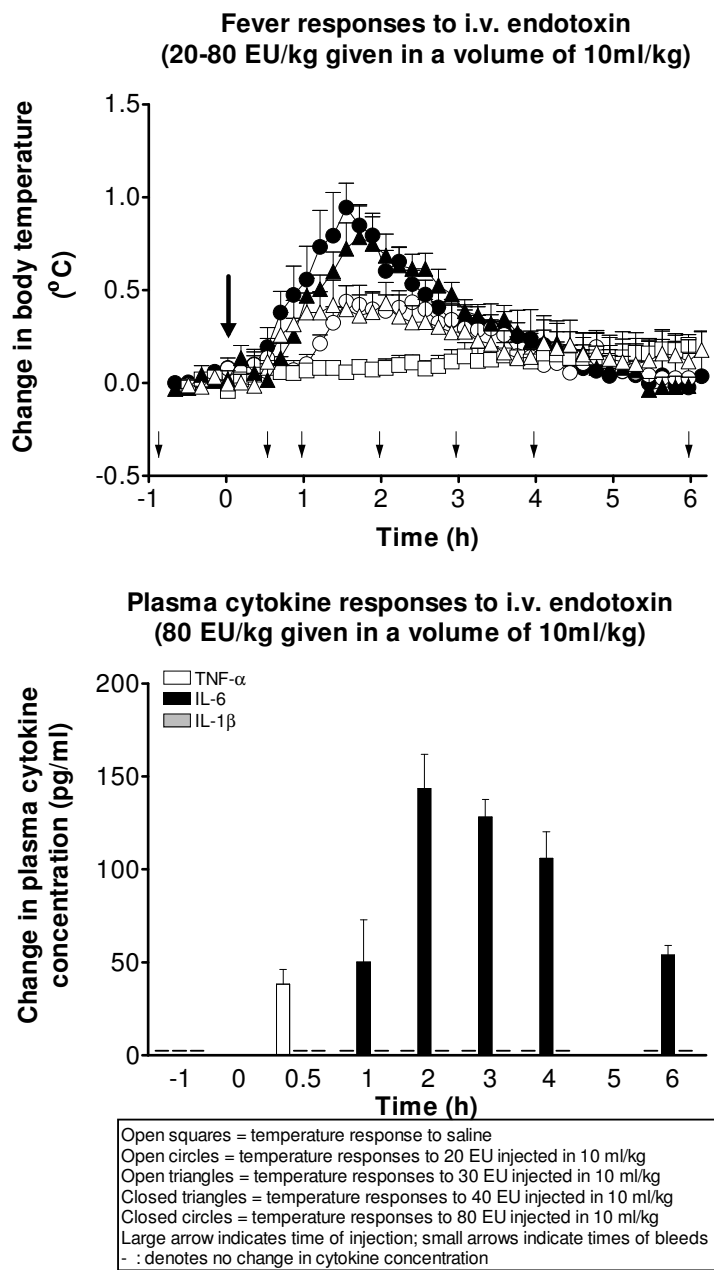


Fig. 1: in vivo challenge of rabbits with different concentrations of LPS from *E. coli* O113: H10

A rise in temperature occurs when injecting 40 EU/kg intravenously. This experiment was the basis for Part 1 of this study, where it was shown whether a similar result could be achieved by challenging rabbit blood in vitro using the same stimulus (see Results), thus proving that the in vitro rabbit whole blood test was an adequate replacement for the in vivo challenge.

2. Literature

2.1. Significance of pyrogens

Pyrogens as fever-inducing substances have been studied extensively over the past 50 years. The concept of fever has been known for a much longer time; as early as 100 years A.D. it was described as a therapeutic remedy by Ruphos of Ephesos. The idea of antipyretic treatments is more recent, and the idea of the therapeutic value of fever has been abandoned. The association of fever with intravenous injections dates from as early as 1642 (Westphal, 1977). In the 18th century, the incidence of fever was attributed to the intravenous injection of putrid material into animals (von Haller, 1757-1766). Hort and Penfeld developed the first rabbit pyrogen test in 1912 and demonstrated that Gram-negative bacteria were pyrogenic whereas Gram-positive bacteria were not. Furthermore, they deduced that the pyrogenic principle was probably a heat-stable bacterial substance. The purified endotoxin principle was first introduced by Boivin and Mesrobeanu in 1932 with a trichloroacetic acid extraction procedure. Since then, more highly purified preparations have been made available (Westphal et. al, 1952). During the second world war, the significance of fever-inducing substances in large volume parenterals such as infusion therapeutics was recognized and extensive research as to the pyrogenic principle and the detection and elimination of pyrogens from intravenously applied drugs has been done ever since.

2.2. Pyrogens

Pyrogens have been divided into two classes: exogenous pyrogens such as endotoxin from Gram-negative bacteria that induce fever when applied intravenously, and endogenous pyrogens that are induced inside the body as a reaction to the contact with exogenous pyrogens and cause an elevation in body temperature. The cells that produce the endogenous pyrogens are mainly the monocytes, a subfraction of the white blood cells. In the body, the endotoxin or lipopolysaccharide binds to a specific receptor

complex on the monocyte containing the so-called toll-like receptor 4. This causes a cascade of reactions leading to the induction of endogenous pyrogens such as Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6) and Tumor Necrosis Factor- α (TNF- α). The Interleukin-8 (IL-8) is also induced in the monocyte by exogenous pyrogens, but is a chemokine by nature with different functions than the other cytokines. The endogenous pyrogens then cause a change in the thermoregulation in the brain towards a higher body temperature. Known pyrogenic substances are endotoxins (LPS, lipopolysaccharide) as components of the cell walls of Gram-negative bacteria, for example the *Escherichia coli* LPS O113: H10, LTA (lipoteichoic acid) as a cell wall component of Gram-positive bacteria, e.g. from *Staphylococcus aureus* (Morath et al., 2002), furthermore peptidoglycan (not included in this study), which are common to both Gram-negative and Gram-positive bacteria, making up 40-90% of the cell wall's dry weight, and the mycoplasmal macrophage-activated lipopeptide (MALP). Other pyrogenic substances comprise bacterial exotoxins, e.g. from *Streptococcus*, enterotoxins, e.g. from *Staphylococcus aureus*, and components of fungi, which cause fever when injected.

All endotoxins have a common general structure. Basically, three regions are distinguishable. The Lipid A portion is covalently attached to the core region which can be divided into an inner and an outer core. The inner core contains a high proportion of sugars that occur in LPS only, for example the 3-keto-deoxy-D-manno-octulosonic acid (Kdo). To the outer core there is usually attached a polymer of repeating saccharide units called the O-polysaccharide or O-chain (O-antigen), typically composed of common hexoses, as is the outer core.

The endless variety of serotypes is due to the almost limitless variability of the O-chain structure (differences in sugars, sequence, chemical linkage, substituents and ring forms). The O-chain is also the part expressed on the surface of the bacterium and is therefore the major target for the host antibody response. The structures in the core region are more limited, whereas the inner core is more conserved than the outer one (identical for example for all *Salmonella* strains).

In 1960, it became clear that the lipid portion contained the pyrogenic activity as mutants lacking parts of the O-chain were found to retain biological activity (Tanamoto et al., 1984). Later, it was found that the biological activity of the Lipid A is determined by the acylation pattern (Rietschel et al., 1993), with up to 7 acyl substituents, the length of those

acyl substituents and the phosphorylation state of the disaccharide backbone (Takeda et al., 1992).

In contrast, the pyrogen LTA binds to the toll-like receptor 2 (Takeuchi et al. 1999) causing a similar cascade and causing fever along the same lines as LPS. For the MALP, an activation of the toll-like receptors 2 and 6 is discussed (Deiters et al., 2003).

The structure of LTA from *S. aureus* comprises a phosphoglycerol backbone esterified with D-alanine (70%) and D-N-acetylglucosamine (15%). Deliberate hydrolysis of the D-alanine substituents revealed a crucial role of those for the biological activity of the LTA, in this case from *Staphylococcus aureus* (Morath et al., 2001).

The need for pyrogen standards was first discussed in the 1950s at a meeting of the WHO expert group. The first ones were preparations from *Proteus vulgaris* and *Serratia marcescens* in 1953, both were evaluated for their potency in the rabbit pyrogen test. *Serratia* turned out to be more potent but was not chosen to be an international pyrogen standard. By 1956, the committee had selected an LPS preparation from *Shigella dysenteriae* to be the first world's pyrogen standard. Later it turned out to be inferior to other standards such as *Salmonella abortus equi* (Novo Pyrexal) or *E. coli* O55 (EC-2). *Escherichia coli* is a Gram-negative bacterium commonly associated with intestinal and urinary tract infections. However, the LPS from *E. coli* alone can be the cause of a number of diseases. Being a gut commensal, the human intestine contains many hundred grams of Colis. Diffusion into the portal vein is being dealt with by Kupffer cells under normal conditions. However, in case of a breakdown of the intestinal barrier, as is the case e.g. in shock, the resulting systemic inflammation can lead to multiple organ failure, disseminated intravascular coagulation (DIC) and death.

E. coli LPS is highly toxic, which can in part be attributed to its structure with a hexa-acyl diphosphorylated Lipid A. *E. coli* O113 LPS is the current WHO reference standard endotoxin preparation provided by the National Institute for Biological Standards and Controls (NIBSC), London.

Salmonella LPS is typically considered to be as active as *E. coli* LPS.

Serratia was long thought to be non-pathogenic. In 1951/2 the US army even conducted an operation called "Sea-Spray" to study wind currents. They burst balloons with *Serratia marcescens* over San Francisco. Later, *Serratia* was associated with pulmonary

and urinary tract infections, as well as endocarditis, osteomyelitis, septicemia, wound and eye infections and meningitis.

Bordetella pertussis, the agent of the whooping cough, possesses a so-called LOS (lipo-oligosaccharide), that is the bacterium only expresses a short non-repeating polysaccharide unit. Since the Lipid A expresses only five acyl chains, a low activity can be expected and has been shown.

Staphylococcus aureus is a clump-forming Gram-positive coccus that, besides being a major cause for nosocomial wound infections and contaminations of indwelling medical devices, can cause food poisoning (enterotoxins) and Toxic Shock Syndrome (TSS) by release of superantigens.

2.3. Mechanism of pyrogenic effects

2.3.1. Binding proteins (BIP, LBP, soluble CD14)

Some two decades ago the association of LPS with host cells resulting in their activation was believed to be based on an interdigitation of the lipid A acyl groups into the membrane of cellular targets (Kabir et al., 1978). As a result of the work of Morrison, Wright, Ulevitch, Tobias and others specific binding proteins and receptors are now known to be involved in the recognition of LPS by the host cell.

The Cluster of Differentiation (CD) 14 receptor, a glycoprotein whose significance was recognized in 1990, exists in a membrane-bound (mCD14, a 53 kDa protein) and a soluble form. The former is embedded in the plasma membrane via a GPI anchor (see below). The latter, sCD14, is capable of inhibiting LPS activity (Schütt et al., 1992), but additionally LPS/sCD14 complexes can induce biological responses in certain CD14-negative cells such as endothelium (Frey et al., 1992). LPS binding takes place via the Lipid A component and is of high affinity (Kirkland et al., 1993).

Several investigators have reported CD14-independent LPS binding at high LPS concentrations (Couturier et al., 1992). In this case LPS may act on an unknown second receptor.

LPS Binding Protein (LBP): LBP is synthesized in hepatocytes as a glycosylated 58 kDa protein which is constitutively secreted into the blood stream (Schumann et al., 1990, Tobias and Ulevitch 1993). The concentration in normal serum is approximately 14-22 µg/ml but can reach a maximum of 200 µg/ml in acute-phase serum (Tobias and Ulevitch,

1993). LPS binding is located in the N-terminal region. LBP shares 44% homology with bactericidal/permeability increasing protein (BPI) (Tobias et al., 1992).

The main known biological activity of LBP is to augment LPS activity. The well-known LPS activity enhancement of serum is at least in part due to the presence of LBP. The enhancing may be due to the LBP singling out LPS monomers from the micelles that the hydrophobic molecule forms. The LBP is capable to transfer LPS to the cellular binding site, the CD14 protein (Hailman et al., 1994). Furthermore, kinetic experiments showed that upon longer exposure to LPS, the LBP mediates diffusion of LPS into HDL, thus neutralizing the endotoxin (Wurfel et al., 1994).

The BPI is a protein of the innate immune system that is only cytotoxic for Gram-negative bacteria and found in polymorphonuclear leucocytes (PMNs). The BPI is highly conserved (> 60% homology) between species and inhibits the growth of a wide range of Gram-negative bacteria without any toxicity for Gram-positive bacteria or eukaryotic cells. The remarkable specificity reflects the strong affinity for the LPS in the bacterial envelope, including that of encapsulated and non-encapsulated bacteria (Elsbach and Weiss, 1992 and Gazzano-Santorio et al., 1992). Upon binding of BPI to the outer membrane, several immediate but reversible effects are manifest, including growth arrest, membrane permeability changes and activation of enzymes degrading phospholipids and peptidoglycans (Mannion et al., 1990). Later, alterations of the cytoplasmic membrane with impaired energy metabolism and irreversible growth arrest, occur. The change from reversible outer membrane damage to irreversible inner membrane changes is accelerated by components of the complement (Mannion et al., 1990) and phospholipase A₂ (Wright et al., 1990).

Binding of BPI to isolated LPS in vitro blocks the ability of LPS to trigger host responses, e.g. the release of cytokines (Ooi et al., 1991, Weiss et al., 1992). Therefore, BPI protects against invading Gram-negative bacteria by blocking bacterial proliferation and inhibiting endotoxin-mediated effects.

2.3.2. Receptors

The innate immune system of mammals uses the family of toll-like receptors (TLR) to engage microorganisms by recognizing so-called PAMPS (pathogen-associated molecular patterns) (Medzhitov and Janeway, 1997). The term TOLL originally referred

to a cell surface receptor governing dorsal/ventral orientation in the early *Drosophila* larvae (Stein et al., 1991). So far, 10 human members of this receptor family have been identified (Akira et al., 2001) and recent years have seen rapid progress in identifying the roles of these receptors (Hayashi et al., 2001, Takeda and Akira 2001, Hemmi et al. 2000). A mammalian homologue of the *Drosophila* Toll receptor was identified in mammals (Medzhitov et al., 1998). TOLL and TLR family proteins are characterized by an extracellular domain with leucine-rich repeats and an intracellular region containing a TOLL/interleukin 1 receptor (TIR) domain. Homologous signalling components have been identified for mammalian TLR and *Drosophila* TOLL, suggesting phylogenetic conservation (Horng and Medzhitov, 2001). Antigen-Presenting Cells (APC) such as macrophages express TLR on their surface, bind PAMP, and initiate a pathway that induces host defense through reactive oxygen and nitrogen intermediates. It also initiates adaptive immunity by inducing proinflammatory cytokines. All TLRs activate a common transduction pathway that culminates in the activation of different factors: the activation of NF κ B (nuclear factor- κ B), the mitogen-activated protein kinases (MAPKs), ERK (extracellular signal-regulated kinase), p38 and the c-Jun N-terminal kinase (JNK). For the recognition of LPS, the TLR4 receptor is crucial (Hirschfield et al., 2000). The role of TLR4 was determined on TLR4 deficient mice which proved to be unresponsive to LPS (Hoshino et al, 1999, Qureshi et al, 1999, Poltorak et al., 1998). Upon binding, the TLR4 receptor dimerizes and the intracellular cascade begins.

LPS binds to LBP and this complex is subsequently recognized by CD14, a glycosylphosphatidylinositol-anchored molecule expressed on monocytes/macrophages and neutrophils. It was long unclear how the CD 14 receptor can be involved in signaling since it does not have a transmembrane portion. It seems likely today that CD14 catalyzes the insertion of the LPS molecules into either the plasma membrane or directly into the receptor complex which consists of the TLR4 receptor and an extracellular protein called MD-2 (Jiang et al., 2000, Shimazu et al., 1999, Akashi et al., 2000). The involvement of the TLR2 receptor in LPS signaling has long been controversial (Yang et al., 1998). Recently, it could be proved by refined purification methods that the TLR2 involvement was due to a contamination of the LPS with lipoproteins and that the TLR4 alone is involved in LPS signaling. Nevertheless, interesting exceptions exist, for example the LPS from *Leptospira interrogans*, which is recognized by TLR2 (Werts et al., 2001). The MD-2 molecule is an extracellular protein without a transmembrane portion that is necessary for binding of LPS (Akashi et al., 2000; Shimazu et al., 1999). Its role could be

either holding the LPS in place for TLR4 pattern recognition or promote the dimerization of the TLR4 receptor through conformational change. How the complex of CD14, TLR4 and MD-2 interacts with the LPS is so far unknown.

After binding, the LPS is rapidly delivered into the cytoplasm. A candidate molecule for the intracellular recognition of LPS is the protein Nod1 which induces activation of the transcription factor NF κ B (Inohara et al., 2001).

The TLR2 receptor recognizes a variety of microbial components such as lipoproteins from mycoplasma, for example MALP-2 (Takeuchi et al., 1999, 2000, 2001), Peptidoglycan and LTA from Gram-positive bacteria and lipoarabinomannan, a cell wall component of *Mycobacterium* (Jones et al., 2001). Ligand recognition of such a wide variety involves cooperation with the TLR1 and -6 (Ozinsky et al., 2000, Takeuchi et al., 2001), which share a 69% amino acid homology with each other and are therefore counted to the TLR2 subfamily (additionally TLR10). TLR2 and -6 cooperate to recognise microbial lipopeptides. TLR6-deficient mice showed no response to MALP-2, whereas the response to bacterial lipopeptides was normal (Takeuchi et al., 2001).

2.3.3. Intracellular signaling

The cytoplasmic domains of the *Drosophila* Toll receptor and the mammalian IL-1 β receptor are highly conserved and referred to as Toll/IL-1 receptor (TIR) domains. Accordingly, the signaling pathways of the toll-like receptors and the IL-1 receptor family are highly similar. Both interact with the adaptor protein MyD88 (myeloid differentiation factor), which has a TIR domain on its C-terminal portion and a death domain on its N-terminal portion.

MyD88 associates with both the TLR4 and the IL-1 β receptor via interaction of their respective TIR domains (Takeuchi et al., 2000). Upon stimulation, MyD88 recruits serine/threonine kinases of the IRAK (IL-1 receptor associated kinase) family (Wesche et al., 1997). These then act through TRAF-6 (TNF-receptor associated factor) to promote both MAP kinase cascades and the NF κ B-inducing cascades (Underhill and Ozinsky, 2002). In the latter, TRAF-6 activates two kinases (Ik α and β) to form a dimer (IkK) that phosphorylates an inhibitory protein, I κ B. The latter is associated to the transcription factor NF κ B, inhibiting it. Upon phosphorylation, the I κ B dissociates, is degraded and the

NfκB enters the nucleus. The transcription of proinflammatory cytokines is initiated. The same holds true for the transcription of the IκB, which then re-inactivates NfκB.

Evidence is accumulating though, that LPS signalling may occur through alternative, MyD88-independent pathways. NFκB activation is merely delayed in MyD88 deficient cells, but completely abolished in TLR4 deficient cells (Kawai et al., 1999). A recently discovered protein, TIRAP (or MAL) activates NFκB when overexpressed, associating with IRAK-2 (Fitzgerald et al., 2001, Horng et al., 2001). In 2002, its role in the MyD88-dependent pathway was determined (Yamamoto et al., 2002). MyD88-independent activation may involve nuclear translocation of Interferon (IFN) regulatory factor (IRF)-3 (Kawai et al., 2001). Recently, an additional molecule involved in TLR signalling was found called RICK (alternatively Rip2 or Cardiak) (Kobayashi et al., 2002).

So far, only very little has been published on species differences in the activation of TLR. Only few sequences are available, most of them TLR4 (mouse, dog, cat, rat) and TLR2 (chicken). Human and rodent cells respond differently to LPS, a specificity conferred mainly by the TLR4 (Lien et al., 2000; Poltorak et al., 2000). This seems to be due to a hypervariable region (Kawasaki et al., 2000). Chicken tlr2 covers both the response to LPS and lipoproteins indicating an increasing complexity of the toll-receptor family in evolution (Fukui et al., 2001).

2.4. Cytokines

2.4.1 IL-1β

The production of IL-1β has been demonstrated in nearly all tissues containing mononuclear phagocytes, including blood (monocytes), lung (alveolar macrophages), (Simon and Willoughby 1981 and Atkins et al. 1967) liver (Kupffer cells), (Dinarello et al. 1968), bone marrow, (Lee et al. 1981), spleen (Atkins et al., 1967) and umbilical cord/placenta (Dinarello et al., 1981 and Flynn et al, 1982). The IL-1β exists in the mononuclear cells as a preformed molecule, the pro-IL-1β. Upon stimulation, a protease is activated, the IL-1 Converting Enzyme (ICE), whose catalytic function is essential for the generation of mature, extracellular IL-1β (Thornberry et al., 1992). The IL-1β family of proteins comprises the agonists IL-1α and IL-1β and the antagonist IL-1ra, which act with equal high affinity upon the receptors IL-1RI and IL-1RII. The IL-1 receptor antagonist (IL-1ra)

is a unique, naturally occurring ligand lacking one of the three binding epitopes of the agonists.

Upon binding to IL-1 β , the IL-1RI associates with the IL-1RacP (accessory to IL-1RI) (Greenfeder et al., 1995). IRAK is then recruited to the complex and autophosphorylated (Cao et al., 1996). IRAK and TRAF-6 then form a complex activating the NF κ B (see above) (Cao et al., 1996).

The final role of IL-1 β in the generation of fever has yet to be determined. The most direct evidence of its involvement comes from studies where recombinant IL-1ra was injected. Fever reaction after administration of LPS was significantly attenuated (Luheshi et al., 1996, Smith et al., 1992) as well as the IL-6 increase in plasma levels which normally parallels fever (Smith et al., 1992, Luheshi et al., 1997). Controversial studies exist as to the pathway by which circulating cytokines induce fever. Banks et al. have published on this topic in 1991 and 1994, showing the existence of transport mechanisms in the blood-brain barrier whereby cytokines can enter the brain from the periphery. Other hypotheses have been proposed, including the passage of cytokines via brain areas devoid of a blood-brain barrier, especially the OVLT (organum vasculosum laminae terminalis) (Blatteis et al., 1992) or activation of cytokine receptors on the blood side of the brain which in turn transduce a signal via other mediators (Van Dam et al., 1996). Anyway, substantial evidence exists that cytokine action is pivotal to the generation of fever (Rothwell and Hopkins 1995). Direct injection of IL-1 β into the brain induces increases in body temperature (Rothwell et al., 1990). Studies have demonstrated an inhibition of the increase of hypothalamic IL-6 after application of a neutralizing antibody to IL-1 β suggesting a secondary role of IL-6 (Klir et al., 1994). The importance of brain IL-6 in fever was further demonstrated by Chai et al., 1996.

It is established that most cytokines induce fever via the release of other mediators, notably prostagandins and the release of corticotropin-releasing factor (CRF). Several studies show that IL-1 β induces the expression, synthesis and release of CRF (Tsagarakis et al., 1989, Suda et al., 1990, Lee and Rivier, 1994).

2.4.2. IL-6

IL-6 belongs to the IL-6 cytokine superfamily. All members of the family employ the gp130 receptor subunit for signalling. Binding of a ligand to this receptor results in homodimerization and activation of receptor-associated kinases, the Janus-kinases (JAKs). These JAKs in turn phosphorylate cytosolic proteins called signal transducers

and activators of transcription (STATs), causing them to dimerize through interaction of their respective Src homology 2 (SH2) domains. Dimerization probably exposes a nuclear localization signal (NLS), allowing the dimer to translocate to the nucleus and activate genes (Guschin et al., 1995). The IL-6 family, for instance, activates STAT 3 via the JAKs 1, 2 and TYK 2 (Leonard 1996).

2.4.3. TNF- α

TNF- α acts as a homotrimer on 4 receptors, two of which are involved in the inflammatory and acute phase response of the organism, CD 120a (p55, TNF-Receptor 1) and CD 120b (p75, TNF-Receptor 2). Obliteration of both results in deficient defense against certain pathogens and reduced inflammatory response (Rothe et al., 1993, Erickson et al, 1994). CD 120a carries an intracellular death domain (DD) through which it binds to the DDs of intracellular adaptor proteins such as TNF-receptor associated death domain (TRADD), receptor interacting protein (RIP) and RIP associated ICH-1/CED-3 homologous protein with death domain (RAIDD) which continue the signalling cascade downstream (Hsu et al., 1995, Stanger et al., 1995, Duan and Dixit 1997). Apart from that, the membrane-proximal region of the intracellular portion of CD 120a can participate in signalling both in cooperation with and independently of the DD. CD 120b does not carry a DD and associates with the protein TRAF2, an enzyme through which the receptor initiates signalling.

Effects of engagement of the above receptors are

- activation of the caspase cascade, a crucial event in apoptosis. ICE, interleukin-1 converting enzyme, a caspase, cleaves pro-IL-1 β into its active form.
- activation of phospholipases, namely phospholipase A₂, which generates arachidonic acid, which in turn is the substrate for inflammatory lipid mediators such as the prostaglandins
- activation of protein kinases; these make up three known cascades, one of which leads to the upregulation of the transcription of IL-6 and IL-8

2.4.4. Effects of cytokines

The proinflammatory cytokines IL-1 β , IL-6 and TNF- α have many overlapping functions referred to as redundancy. Therefore, many partially contradicting publications can be found as to which one plays the key role in the pathological effects in the organism.

TNF- α is probably the most extensively studied cytokine of the three. It is often attributed the key role of proinflammatory effects in the body.

All are pleiotropic cytokines, meaning that they act on many tissues simultaneously. They orchestrate the reaction of the organism to infection and/or pyrogenic challenge. In the bone marrow, the three inflammatory cytokines induce an increased neutrophil mobilization. In the hypothalamus, the body temperature is increased, at the same time protein and energy mobilization is taking place in order to enable the body to provide the energy for the fever. TNF- α in addition enhances the migration of dendritic cells to the lymph nodes, where they may present their antigens to naïve T-lymphocytes.

On the level of endothelial cells, cytokines induce a shift in the adhesion molecules expressed on the endothelial cell wall towards molecules that have a higher affinity. Within minutes after exposure, the cells express P-selectin, which binds to sulfated sialyl lewis on the surface of neutrophils, causing them to slow down and adhere to the endothelium. A switch to E-selectin is taking place within 2 hours. While the resting endothelium is expressing low levels of ICAM-2 (intercellular adhesion molecule) after exposure to TNF- α there is a switch to ICAM-1 which binds to the LFA-1 (leukocyte functional antigen) on monocytes and polymorphonuclear cells. The structure and affinity of the molecules towards each other is also influenced by IL-8 (see below). All that induces phagocytosing cells to find the site of infection, extravasate and eliminate the infectious agent.

Furthermore, local infection causes a cytokine-induced vasodilation with ensuing extravasation of proteins and white blood cells. In the case of a systemic infection, as in septic shock, however, the same mechanisms can cause hypovolemia with ensuing hemoconcentration, hypovolemic shock, DIC (disseminated intravascular coagulation) and multiple organ failure.

2.5. IL-8

Intracellular pathways regulate the extent and duration of IL-8 gene expression. Regulation of the IL-8 promoter is well understood: its activation integrates at least three signal transduction pathways (Holtmann et al., 1999). It contains binding sites for the transcription factors NF κ B, AP-1 and C/EBP (also called NF-IL-6) (Mukaida et al., 1989). A functional NF κ B binding site is essential for the induction of the promoter by LPS or IL-1 β . For maximal gene expression two more signals are needed. These additional signals involve AP-1 or p38 MAPK. AP-1 consists of a dimer composed of

Fos, Jun or ATF-2. It is activated via protein kinases including ERKs or SAPK/JNK. The MAP-kinase p38 is involved in pathways known to stabilize mRNA (Hoffmann et al., 2002), whose degradation is modulated via AU rich elements (ARE) in its 3' untranslated region. NF-IL6 is not essential for the induction of the IL-8 transcription, but it is needed for maximal activity (Hoffmann et al., 2002, Holtmann et al., 1999). LPS from *Salmonella abortus equi* and LTA from *Staphylococcus aureus* are known to be potent inducers of IL-8 (von Aulock et al., 2003, Standiford, T. J. et al., 1994, Blease et al., 1999., Zhong et al., 1995). Since a different capacity of LPS and LTA to trigger IL-8 release was described (von Aulock et al., 2003) it needs to be elucidated to which extent these stimuli activate the pathways mentioned above.

2.5.1. Effects of IL-8

Chemokines signal through an ancient and highly conserved family of receptors, the seven-transmembrane-domain, trimeric G protein coupled receptor family. All chemokines use a GTP binding protein called G-Protein, of which 20 have so far been identified. All of them are made up of the three subunits α , β and γ .

When the receptor, the fMLP receptor, binds its ligand, a change in structure allows it to bind to the G-Protein. The resting G Protein is associated with GDP, which upon activation is replaced by GTP. The G Protein now dissociates into two subunits, G α and the dimer G $\beta\gamma$, each of them capable of transmitting/amplifying signals. Binding of the alpha subunit to its ligand activates the inherent GTP-ATPase activity of this subunit, cleaving the molecule to GDP and allowing the subunits to reassociate.

Chemokines play an important role in the response of the innate immune system to various pathogens. To date about 50 human chemokines have been identified and the family is still growing. Interleukin 8 (IL-8), originally called monocyte-derived neutrophil chemotactic factor, was the first member characterized. IL-8 is an 8 kDa cytokine and belongs to the CXC-subfamily. The chemokine IL-8, a monomeric chemokine (Burrows et al., 1994) included in this study, is produced by monocytes, macrophages, fibroblasts, keratinocytes, T-cells and endothelial cells. It is a so-called CXC chemokine binding to the according receptors CXCR-1 and CXCR-2 (Moser et al., 1993) which are expressed on neutrophils (Rajaratnam et al., 1994), and, to a lesser extent, on monocytes, eosinophils and basophils. Both trigger changes in the Ca^{2+} content of the cytosol and induce chemotaxis and granule exocytosis, CXCR-1 alone induces a

respiratory burst and phospholipase D activation (Jones et al., 1996). Its functions are to arm neutrophils, making them capable to produce the respiratory burst generating oxygen radicals, an important tool in innate immunity, and to release their lysosomal contents. Another important function is the conformational change of the LFA-1 (leukocyte functional antigen) and the MAC-1, also called CD 11b/CD18, both on white blood cells. This change increases the affinity to the ICAM-1 considerably, causing the white blood cells to adhere to the endothelium and extravasate. IL-8 binds to the proteoglycans of the endothelial matrix and therefore forms a gradient to the site of infection and indicating the site of extravasation.

2.6. Pyrogen measurement

2.6.1. Rabbit pyrogen test

The rabbit pyrogen test has been the gold standard in pyrogen testing since 1942, when it was introduced into the USP (United States Pharmacopoeia). The rabbit species was chosen by Seibert, who also discovered the pyrogenic principle (Seibert, 1925). This choice proved to be fortuitous, as the fever reactions of e.g. rats, mice, hamsters and chicks is irregular and unpredictable. It involves a measurement in body temperature after the application of not more than 10 ml/kg bodyweight of the substance to be tested. The rabbit has a labile thermoregulation and tends to give false-positive results. Also, the very rigid fixation and the handling (injection procedure) can cause a hyperthermia due to excitement. On the other hand, it has been reported that the fixation and lack of movement can cause a hypothermia yielding false-negative results (Grant, 1950). The dog, on the other hand, has a more stable thermoregulation but is reported to be less sensitive to endotoxins than the human (Co Tui, 1942). In 1941, the need for pyrogen testing of LVP (large volume parenterals) due to World War II caused the Committee of Revision of the USP to authorize the first USP collaborative study of pyrogens with pyrogen filtrates of *Pseudomonas aeruginosa*. The study included 3300 rabbit tests with 253 rabbits, 1782 tests with pyrogenic material and 1017 tests with non-pyrogenic saline solution. The results of this study led to the incorporation of the rabbit test in the 12th edition of the USP in 1942. In its simplest form, the test involves measuring a rise in body temperature following intravenous injection of a test solution. Temperature is to be measured by a clinical thermometer or probe inserted into the rectum of the rabbit at a depth of not less than 7.5 cm. The test is limited to substances that can be injected in doses of not more than 10 ml/kg body weight within a period of not more than 10

minutes. A total of three rabbits is injected into the marginal ear vein, the time of injection is recorded and temperatures are read at 1, 2 and 3 hours afterwards. The test is positive if one rabbit in three shows a rise in body temperature of 0.6°C or more than the control temperature or if the sum of the rises exceeds 1.4 °C. In either case, a repetition is performed with an additional 5 rabbits. In this case, a rise of 0.6°C in more than 3 rabbits or a sum of 3.7°C of all eight classifies the substance as pyrogenic.

Comparisons between the reactivity of humans and rabbits in vivo by Greisman 1969 showed that the threshold towards three endotoxin preparations was comparable, but that the humans respond more vigorously than the rabbits (Greisman and Hornick, 1969).

Rabbit breeds used for testing are New Zealand Whites, Belgium Whites, Chinchillas and Dutch Belts. Animals of one single sex are preferred, and there have been reports about male rabbits being more sensitive to pyrogens than females.

The USP has only two requirements for the rabbits as test animals, that they must be both healthy and mature. Maturity is specified by Probey and Pittman 1945 as a weight between 2000 and 3500 g, giving a much more uniform response than rabbits of a weight of less than 2000 g (Tennant and Ott, 1953).

The USP specifies the frequency of use, not the length. Frequency is not to be more than once every 48 hours, or less than 2 weeks following a maximum rise of 0.6°C or more or a testing of substances that were classified as pyrogenic. On the one hand, a reuse of the animals is advisable since the animals get used to the handling and accuracy of testing increases. If on the other hand, antigenic substances are tested, reuse is limited to three times within 7 days, provided no pyrogenicity is detected.

Further requirements of the USP concern maintenance and housing as well as sham testing prior to pyrogenicity testing including all the handling steps except injection. Tolerance to endotoxins was first recognized by clinicians producing experimental temperature elevation in human subjects. Early tolerance in the rabbit has been demonstrated to occur within hours after injection, but is gone within 48 hours. It has been shown not to be specific for the given endotoxin and is due to a hyporesponsiveness of the immune cells. In contrast, the second or late phase is seen after 72 hours and may persist for several weeks. In addition, it is a specific tolerance caused by antibodies directed against the O-antigens and the core carbohydrates of the endotoxin (Greisman et al., 1969).

For the comparison of the in vivo situation with the results of the in vitro rabbit pyrogen test we assumed that a mature rabbit has 40-50 ml blood/kg bodyweight (Mott, 1967).

Recently, a study involving 171 rabbits performed at the Paul-Ehrlich-Institute (PEI) indicated that the in vivo fever threshold of the most sensitive rabbits is at 50 pg/ml (0.5 Endotoxin Units/ml) of the international WHO standard from *E. coli* O113:H10 (Hoffmann et al., 2005). A challenge of 5 EU/kg applied in 10 ml/kg intravenously evoked a fever reaction in 50 % of the rabbits. All rabbits reacted when challenged with 20 EU/kg. This fever threshold of 0.5 EU/ml was chosen as the concentration that has to be found by the human whole blood assay (2.6.3.) which aims to replace the in vivo rabbit test.

2.6.2. LAL (Limulus amoebocyte Lysate Test)

When in contact with the lipid A portion of endotoxin, the amoebocytes from *Limulus polyphemus* (horseshoe crab) coagulate due to an enzymatic reaction (Levin and Bang, 1964). In the presence of calcium, the clotting enzyme zymogen is activated by a serin protease and acts on coagulogen, a clottable protein in the lysate, producing a smaller clot protein. The clotting can be observed by turning the tube with the lysate by 180° (clot endpoint LAL) or, in a more quantitative way, by the turbidimetric LAL, which measures quantitatively time of the clotting. The basic principle has been improved and modified in many ways (Yin et al, 1972). A sensitivity of 0.0005 ng/ml was described by the developers.

The lysate is prepared by placing the crabs in restraining racks and inserting a needle through the muscular hinge between the cephalothorax and the abdominal region. Hemolymph is then drawn from the cardiac chamber into a container with anticoagulant. After collection, the amoebocytes are centrifuged and the supernatant is discarded. After 2-3 washing steps, the cells can be subjected to osmotic shock by adding distilled water and the intracellular lysate is released. Other methods exist, though. The product is then lyophilized and remains stable for ca. 3 years.

One of the drawbacks of the LAL is that it only detects endotoxin. Contaminations of drugs with Gram-positive bacteria or their fragments, e.g. with *Staphylococcus*, are not an unlikely event. Another one is that it does not distinguish between different potencies of LPS preparations, which can differ vastly in the whole blood of a mammal. Therefore, e.g. the LPS from *Pseudomonas aeruginosa* is far more potent in the LAL than it is in the human/rabbit whole blood test.

2.6.3. Whole blood pyrogen test

2.6.3.1. Whole blood incubation

A new way of measuring pyrogens has been introduced in 1995 by Hartung and Wendel (Hartung and Wendel, 1995). Basically, human blood is diluted in physiological saline and brought together with pyrogens or drugs suspected of being contaminated. In the case of pyrogenicity, the monocytes are challenged to produce cytokines in vitro, and the cytokines can be measured by specific ELISAs. The reaction is quantitative, that is, the more pyrogens are in the sample, the more cytokines are produced, and the test is performed in the relevant species, that is the reaction of man is tested. The human whole blood test has in the meantime been successfully introduced and aims to replace the in vivo rabbit pyrogen test.

2.6.3.2. ELISA technique

The ELISA (Enzyme-Linked-Immunosorbent Assay) is an assay based on the reaction of specific antibodies towards an antigen, in this case IL-1 β , IL-6, TNF- α and IL-8. An antibody is bound to a microtiter plate with high protein binding capacity; the pyrogen-stimulated plasma or supernatant are added to the antibody and the cytokine binds. After a washing step, a second, labeled detection antibody is added which also binds to the cytokine; the label is in this case biotin, which binds with high affinity to POD (horseradish peroxidase). After a second washing step, substrate, in our case TMB (Tetramethylbenzidine) is added. The enzymatic reaction of the POD with the TMB changes the color of the latter from colorless to blue and the antibody-antigen binding is made visible.

2.6.4. WEHI assay

The WEHI (Walter and Elizabeth Hall Institute) 164 cell line was introduced in 1973 by Rollinghoff and Warner. It is a murine cell line consisting of fibrosarcoma cells that are sensitive to TNF- α . When in contact with this cytokine, cells undergo apoptosis in a concentration-dependent manner (Gearing et al., 1994). The survival rate of the cells can be made visible with Alamar Blue which is reduced by Cytochrome and e.g. NADPH/NADP, FADH/FAD resulting in a color change which correlates to the level of TNF- α in the plasma in question. The reaction is not necessarily species-specific, that is the murine cells undergo apoptosis also when brought into contact with human or canine TNF- α .