In this work we have studied the acute phase protein response and degranulation of polymorphonuclear leukocytes in vivo in the rat after a slow interleukin-1β stimulation. A total dose of 1 µg, 2 µg, 4 µg and 0 µg (controls with only vehicle) of interleukin-1β was released from osmotic minipumps over a period of 7 days. The pumps were implanted subcutaneously. A cystic formation was formed around the pumps that contained interleukin-1β whereas no tissue reaction was seen around pumps containing only vehicle. Besides fibroblasts the cyst wall contained numerous polymorphonuclear leukocytes which were positively stained for cathepsin G, α2 macroglobulin, α1-inhibitor-3, α-proteinase inhibitor, albumin and C3 were measured by electroimmunoassay and all showed plasma concentration patterns that were dose-dependent to the amount of interleukin-1β released. Fibrinogen in plasma was elevated in the control group but showed decreased plasma values with higher doses of interleukin-1β released. All animals showed increased plasma levels of cathepsin G but the lowest levels for cathepsin G were seen for the highest interleukin-1β dose released. It was clearly seen that a slow continuous release of interleukin-1β in vivo caused an inflammatory reaction. Plasma levels for the proteins analysed all showed a similar pattern, namely an initial increase or decrease of plasma concentration followed by a tendency to normalization of plasma values. It was concluded that a long-term interleukin-1β release could not sustain the acute phase protein response elicited by the initial interleukin-1β release.

Key words: Acute phase response, α1-inhibitor-3, α2 macroglobulin, Cathespin G, C3, Interleukin-1, Osmotic mini pump, Polymorphonuclear leukocytes, Proteinase inhibitors, Proteinases

Introduction

An inflammatory reaction may be defined as 'the reaction of vascularized living tissue to local injury'. The local tissue injury may be caused by mechanical trauma, radiation, microorganisms (infection), neoplasia, burn injury, chemical irritation, etc. Regardless of how the local tissue injury is caused, the vascularized living tissue will react mainly in a uniform way. This is probably due to the different specialized inflammatory cells and their capability to communicate. By inflammatory mediators and cellular receptors, cells are signalling and receiving signals in a huge complex network. Interleukin-1 (IL-1) is a well-known inflammatory mediator holding a central position in the inflammatory reaction. It has the capability to induce an acute phase protein response from the liver and to induce degranulation of polymorphonuclear leukocytes (PMNs) in vivo. Several plasma proteins are increased or decreased as a part of the acute phase response and this acute phase protein response differs from species to species.

Two proteinase inhibitors, namely α1-antichymotrypsin and α1-proteinase inhibitor (α1PI), are among the strongest reacting acute phase proteins in humans. By forming complexes with PMN cathepsin G and elastase they inhibit these two PMN proteases. In the rat α2-macroglobulin (α2M) and α1-inhibitor-3 (α1I3) are marked acute phase proteins. The objective of the present work was to investigate the effects of a slow continuous stimulation of IL-1 on some acute phase proteins and PMNs degranulation in vivo.

Materials and Methods

Assays: Rat cathepsin G was measured by a specific enzyme-linked immunosorbent assay (ELISA) as described. Rat α2M, α1I3, C3 fibrinogen, α1PI and albumin were measured by electroimmunoassay. Antisera against rat α2M and α1I3 were prepared as described. Rabbit anti-rat C3 and rabbit anti-rat fibrinogen were obtained from Cappel, USA and rabbit anti-rat α1PI and rabbit anti-rat albumin were...
a gift from Dr C-B. Laurell (Department of Clinical Chemistry, Malmö General Hospital). Enzymatic activity of cathepsin G was determined using the substrate Suc-Ala-Ala-Pro-Phe-pNA (SucAAP) (Sigma) as described.\textsuperscript{10}

**Immunohistochemistry.** Formalin fixed tissue sections were embedded in paraffin, cut and mounted on glass slides. Staining was done by the peroxidase–antiperoxidase method as described.\textsuperscript{11}

**Animal experiments.** Female Wistar rats (Mollegaard Avelslaboratorium A/S, DK-4623 Skensved, Denmark) weighing 230 g were used. The animals were anaesthetized by an intraperitoneal injection of Mebunal\textsuperscript{10} and then the back of the animals were shaved and disinfected. Through a small incision (approximately 15 mm in length) in the skin an osmotic minipump ‘Model 2001’ (Alzet) was implanted subcutaneously. The flow rate of the pump was 1 μl/h for 7 days. After implantation the rats were allowed to wake up. Four groups of rats containing five animals each received pumps with different amounts of recombinant human IL-1β (rhIL-1β), 0 μg (controls), 1 μg, 2 μg and 4 μg. In addition, two rats received minipumps subcutaneously containing 4 μg IL-1. After 3 days these two latter pumps were removed and reimplanted in two new rats which were followed for 3 days in an attempt to test the functional stability of rhIL-1β during the experimental conditions. The rhIL-1β was prepared by recombinant DNA technology in *Escherichia coli* (Synergen Inc., USA). The protein was diluted to appropriate concentrations in sterile filtered phosphate buffered saline, pH 7.4 (Dulbecco) with 0.2% (w/v) bovine serum albumin (Sigma) and filled into the pumps. Blood samples of approximately 0.4 ml were taken from the tail into tubes containing EDTA. Plasma was immediately prepared and frozen at −70°C. Samples were collected at time 0, just before the implantation of the osmotic minipumps and thereafter every 24 h for 7 days. After 7 days the animals were sacrificed with an overdose of Mebunal\textsuperscript{10} and the eventual cystic formation and cystic fluid around the pumps were collected. The cystic formations were fixed in phosphate buffered formalin until paraffin embedded and the cystic fluids were centrifuged and frozen at −70°C until analysed. During the whole experimental time the animals had free access to water and standard pellet

FIG. 1. Using subcutaneously implanted osmotic minipumps rats were stimulated by a slow release of different amounts of IL-1β diluted in 0.2% BSA (w/v) in PBS. One group received only vehicle (control group), while three other groups received 6 ng/h IL-1β, 12 ng/h IL-1β and 24 ng/h IL-1β, respectively. Here results from the control group (△-△) and the group that received 24 ng/h IL-1β (■-■) are presented. Blood samples were taken from the tail just before implantation of the osmotic pump and thereafter for 7 days. Values are given as mean values ± standard error of the mean. All groups contained five rats. α1M (a), α2PI (b), α1AIP (c), albumin (d), C (e) and fibrinogen (f) were measured by EIA. Plasma cathepsin G (g) was measured by a specific ELISA.

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Results

Tissue reactions: The rats did not show any outer signs of discomfort throughout the experiment. At autopsy a cystic formation was seen around osmotic minipumps containing IL-1 irrespective of dose, whereas no tissue reaction was seen around pumps without IL-1 (controls). No enzymatic activity of cathepsin G as measured by activity against SucAAPP could be detected in the cystic fluid. Cathepsin G concentration in the fluids was measured by a specific ELISA and mean value ± SEM in the groups were: 1 µg IL-1 = 179 ± 7 µg/l, 2 µg IL-1 = 179 ± 13 µg/l and 4 µg/l = 176 ± 8 µg/l.

Acute phase proteins: αM, α1, C3, fibrinogen, αPI and albumin were all measured by electroimmunoassay. αM was not detectable in plasma at time 0, just before the implantation of the osmotic minipumps, when measured by electroimmunoassay. Detectable amounts were seen after one day and throughout the observation period with peak values on day 1 and 2 (Fig. 1a). A dose–response pattern was seen for αM. Plasma αPI showed the highest values on day 2 with values ranging from 140 to 160% of the starting levels (Fig. 1b). The values then gradually decreased during the experimental time. αI, plasma levels decreased with the greatest decrease in the group that received 4 µg IL-1 (Fig. 1c). Normal plasma level was not totally restored during the observation period. The same pattern was seen for albumin (Fig. 1d). Plasma values for C3 were

food. The animal experiments were sanctioned by the local ethical committee for animal experiments.

FIG. 1. Continued.
increased during the whole experimental time (Fig. 1c). Rats treated with the highest doses showed a value of 180% on day 7 compared with the starting value. After an initial increase of fibrinogen in the controls the values thereafter decreased. However, in the two groups receiving the largest amounts of IL-1 an almost immediate decrease in plasma values were seen (Fig. 1f). An equal increase in $\alpha_v$M plasma levels were seen in the two rats that received a pump formerly implanted for 3 days in two other rats (Fig. 2).

**Plasma cathepsin G in IL-1 stimulated rats:** Plasma levels of cathepsin G at time 0 were below the detection level 1.5 $\mu$g/l of the specific ELISA used. Irrespective of the IL-1 dose administered, a biphasic curve was seen in plasma levels (Fig. 1g).

**Immunohistochemistry:** In sections from the cystic formation with adjacent skin numerous inflammatory cells were seen in the dense wall of fibroblasts (Fig. 3a). Approximately half of the inflammatory cells were stained for cathepsin G.

**Discussion**

IL-1 is an important cytokine released in the inflammatory process. There are two forms of IL-1, IL-1$\alpha$ and IL-1$\beta$. They both act on the same receptors and they also elicit the same cellular response. Recently a specific IL-1 receptor antagonist (IL-1ra) has been isolated in its native form and also cloned. The importance of IL-1 in the acute inflammatory process...
b

FIG. 3. Continued.

c

FIG. 3. Continued.
may be illustrated by survival of rabbits receiving a normally lethal endotoxin dose after treating the animals with intravenous injections of IL-1ra. Although the effects of IL-1 are well documented most of the knowledge has come from studies in vitro and studies in vivo may be a valuable complement for the understanding of the pathophysiological role of IL-1.

In this work we have studied the effects on the acute phase protein response and the degranulation of PMNs by a slow IL-1 release stimulation over a period of 7 days. Even with only vehicle present in the mini osmotic pumps (control group) a clear inflammatory reaction could be seen for the acute phase proteins. In these animals fibrinogen, α1PI and α2M all showed elevation of plasma values for the first 2 days but these values decreased over time towards the origin values. C3 in plasma increased to 125% after 2 days and thereafter the plasma values stayed at this level throughout the observation time. Both albumin and α1I showed decreased plasma values over the whole observation period. Cathepsin G was released to the bloodstream as measured by elevated plasma levels from the second day to the seventh day. All these findings are in agreement with an inflammatory reaction due to the trauma from the operative procedure. No local tissue reaction was seen around the implanted pumps only containing vehicle, indicating that no greater irritation was caused to the surrounding tissue.

When IL-1 was released from the mini osmotic pumps a more pronounced acute phase protein reaction was seen. For α2M, α1PI and C3 there was a dose-dependent pattern seen in the elevated plasma values. Also for the negative acute phase protein reactants albumin and α1I, a dose-dependent pattern could be detected. Fibrinogen is a positive acute phase protein reactant in humans reaching a maximum plasma concentration approximately on the third day after tissue injury. When increasing amounts of IL-1 were given the plasma levels of fibrinogen decreased over time and the positive acute phase response was changed into a negative acute phase response. How this change in acute phase answer is mediated is not known but our results are in agreement with a study by De Jong et al.

The data so far discussed clearly show that the tissue injury caused by implantation of the osmotic minipump is causing an acute phase protein response and that this response is enhanced by the stimulation of IL-1β. The general plasma protein levels over the experimental time showed an initial peak or dip followed by a tendency to return to original values in spite of the continuous release of IL-1 over the whole experimental time. To examine that the IL-1 in the pumps did not lose its biological activity we removed pumps from two rats and reimplemented them in two new rats. The same reaction pattern for α2M was seen in all these four rats clearly indicating that IL-1 did not lose its biological activity over the experimental time. Therefore, an explanation for these results may be that the initial release of IL-1 also provoke a reaction to counteract effects of IL-1. This effect could be mediated by an induction of IL-1 receptor antagonist. IL-1 has the capability to induce IL-6 production which in turn may induce IL-1 receptor antagonist production as recently shown. Another explanation could be inactivation of IL-1 in the immediate surrounding of the pumps by proteolytic enzymes. In this study we showed high levels of cathepsin G in the cystic formations. However, no enzymatic activity against the cathepsin G substrateSucAAPF was detected indicating the measured levels of cathepsin G to be enzymatically inactive probably by inhibition by protease inhibitors or by autodigestion.

Around the osmotic mini pumps cystic formations containing a serous liquid were formed when the pumps contained IL-1, while in pumps with only vehicle no cystic formation was seen. This reaction has previously been described by Lewis et al. and Dunn et al. It is known that IL-1 has the capability to stimulate fibroblasts and IL-1 is therefore suggested to be one of the major mediators of skin inflammation. It is likely that the cystic formation seen is a result of such a stimulation. Cathepsin G was measured in the liquids and values were approximately 170 g/l irrespective of the IL-1 dose given. In the wall of the cystic formation numerous inflammatory cells could be seen in histological sections. About 50% of the cells were stained for cathepsin G which may be a source for the high concentrations of cathepsin G seen in the fluid. Cathepsin G and other proteases like elastase from PMNs have the capability to degrade proteins and cathepsin G and elastase has been shown to be able to degrade TNFα and TNFβ but not IL-1α. The presence of proteases in this cystic formation surrounding the minipump may influence the IL-1 released.

While the acute phase proteins showed a dose-dependent response to the amount of IL-1 administered cathepsin G plasma levels did not show any tendency to a dose-dependent release. These data are in contrast to our previous study where cathepsin G plasma levels showed a dose-dependent pattern to a single injection of IL-1. We have no obvious explanation for the difference in these results. When proteolytic enzymes like cathepsin G are released to the bloodstream, complexes with inhibitors are formed. These complexes are then removed from the circulation by the liver. This removal is mediated by specific receptors on the endothelial cells in the liver. It may be speculated that the capacity of the liver to remove complexes from the bloodstream may be improved due to the long standing IL-1
stimulation and thus the lowest cathepsin G plasma concentrations are measured in the highest IL-1 dose given.

To summarize, we have found the initial release of IL-1 to be the most important stimulation regarding the acute phase protein response from the liver. Although the IL-1 release was continued for 7 days the acute phase protein levels in plasma approached towards normal values after the increase/decrease during the first 2 days.

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