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Early Inflammatory Cytokine and Acute Phase Protein Response Under the Stress of Thermal Injury in Rats

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Summary

The acute inflammatory response associated with thermal injury was examined in rats. The appearance of mediators of inflammation in the systemic circulation, including cytokines interleukin-1 (IL-1), tumor necrosis factor (TNF) and interleukin-6 (IL-6) and acute phase proteins were assessed during initial 72 h following thermal injury. Increased levels of activity were noted for all three cytokines, but with a different time-course. While serum IL-1 activity was elevated throughout the 3-day period of observation, the levels of serum TNF activity were enhanced after 12 h and on days 1 and 3 following scalding injury. The values of IL-6 were already increased one hour after thermal injury and increased progressively up to day 1 following scalding. Alpha$_2$-macroglobulin and haptoglobin levels were increased 12 h after thermal injury, rising further on days 1 and 3. Positive correlation was found between the time-course of increased serum IL-6 activity and alpha$_2$-macroglobulin, as well as between TNF and haptoglobin in the serum..

Key words

Thermal injury • Inflammation • Cytokines • Acute phase proteins

Introduction

Stress factors such as infection, physical or chemical trauma trigger a series of natural defense reactions which constitute the homeostatic process, namely inflammation. The early phase of inflammation is known as the acute phase (AP) response in which local and systemic activation of cells and soluble mediators of inflammation are generated which mobilize the metabolic response of the whole organism (Baumann and Gauldie 1994). Prominent among these changes is the increase in serum levels of acute phase reactants, a group of serum proteins synthesized in the liver, believed to be of particular importance for the adjustment of physiological processes of stress response (Weissman 1990). The common pathway in inflammation initiated by various stress factors concerns the induction of cytokines, regulatory proteins secreted by cells in response to injury, which mediate multiple local and systemic biological functions involved in the response to injury (Smith *et al.* 1992, Cerami 1992). Cytokines interleukin-1 (IL-1), tumor necrosis factor (TNF) and interleukin-6 (IL-6) have attracted special attention as principal cell messengers in the interactions that occur in the host effector response to tissue injury (Smith *et al.* 1992, Kataranovski *et al.* 1998) and as mediators involved in the control of acute phase protein expression (Baumann and Gauldie 1994).

Traumas of sufficient magnitude which cause extensive tissue damage including mechanical, surgical or thermal injury, as well as hypovolemic hemorrhagic

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shock, result in many physiological alterations, These may include hepatic synthesis of acute phase protein, dysfunctional temperature regulation, fever in the absence of infection, hemostatic changes, muscle wasting associated with negative nitrogen balance and hyperglycemia (Wolfe *et al.* 1982). This hypermetabolic response, often called the systemic inflammatory response syndrome (SIRS), concerns excessive whole-body inflammation (Nuytinck *et al.* 1988) and is considered as a major determinant in the development of multiple organ dysfunction, often with a lethal result (Rangel-Frausto *et al.* 1995).

Although it is known that cytokines IL-1, TNF and IL-6 are involved in the hypermetabolic host response to trauma, the exact cytokine response to various traumatic insults is poorly understood. Despite multiple investigations, no consistent pattern of cytokine response to traumatic injury is available, because various kinds of trauma are influenced by various kinds of stress (Abraham 1991) resulting in a different time-scale of postinjury cytokine response.

Thermal injury to the skin is a suitable model of trauma-induced inflammation because it is possible to correlate the severity of injury with the inflammatory response evoked. Thermal injury of the skin is characterized by a loss of the epidermis, blistering, exudation and inflammation of the dermis due to thermal tissue damage (Ward and Till 1990). In experimental animals, thermal injury models make it possible to control the extent of tissue damage by adjusting the area and depth of burn injury (Civil and Schwab 1988). In the present study, we used skin burns in rats to obtain information regarding the systemic cytokine response on an exact time-scale following injury with a special respect to the immediate cytokine response. As the information concerning early development of the acute response phase under the stress of thermal injury is sparse (Ševaljević *et al.* 1988), dynamics of the changes of serum acute phase proteins is also being studied. The time course of changes in the circulating levels of IL-1, TNF and IL-6 as well as in serum concentrations of acute phase proteins alpha$_2$-macroglobulin and haptoglobin (major and moderate acute phase proteins of the rat) were determined during the initial postburn period in rats. In clinical terms, this period is called "shock stage" and usually ends two to three days after thermal injury (Evans 1969). Some parameters of the response to injury, i.e. body temperature and hematocrit, were followed during the same period.

Method

Male 10-week-old albino Oxford rats weighing 200-220 g, bred at the Institute for Medical Research, Military Medical Academy in Belgrade were used. The experimental model of thermal injury described previously (Kataranovski *et al.* 1994) was employed in our study and conducted in adherence to the NIH guidelines for the use of experimental animals, with the approval of the Ethical Committee of our Institute. Briefly, ether-anesthetized rats were placed in the supine position in a plaster cast with an area of their backs exposed through an opening in the cast, immersed into a hot water bath (80 °C) for 30 s. This produced nonlethal, full-thickness injury to skin that covered 20% of the body surface area (Civil and Schwab 1988). Sham-injured animals were anesthetized only.

Rectal temperature was measured with a digital thermometer (Ellab A-S, type DU 3S, Copenhagen, Denmark) with a resolution of 0.10 °C. Hematocrit was determined in whole blood samples in heparinized capillary tubes (Gold Seal Glasware, USA) using a microhematocrit centrifuge (MSE, USA). Records of these parameters were determined in groups of six to eight animals at various time-points (1, 3, 6, 12, 24 and 72 hours) following scalding injury. At these times blood was withdrawn from the abdominal aorta of sham-injured and injured animals (6-8 per group) and the serum was obtained after blood clotting and centrifugation at 5 000 x g for 15 min. Serum samples were examined for the presence of endotoxin in the Department of Drug Control (Hemofarm Vršac, Yugoslavia) by using the *Limulus amebocyte* lysate assay (LAL assay) according to the Euopean Pharmacopeia (test sensitivity 0.03 ng/ml). Serum samples of sham-injured and thermally injured animals (1 to 72 h following scalding) were found to be negative for the endotoxin.

The serum concentration of alpha$_2$-macroglobulin (alpha$_2$-M) and haptoglobin (Hp) were measured by rocket immunoelectrophoresis according to Baumann (1988). Polyspecific rabbit antiserum to rat acute phase proteins was prepared against the serum of rats treated with turpentine, a potent inducer of the acute phase response in rats (Kushner 1982). The antibody to rat alpha$_2$-M was isolated from rabbit antisera directed against rat turpentine sera, as described by Zečević *et al.* (1973). For the haptoglobin determination a monospecific antibody to human Hp was used (N-antiserum to human haptoglobin, Behring, Marburg, Germany), which was

cross-reactive with rat haptoglobin. The relative concentrations of alpha$_2$-M and Hp were established by quantification of the areas under the respective immunoprecipitation peaks. The concentration of albumin was measured with an ASTRA-8 (Automated Stat/Routine Analyzer, Beckman). The results are expressed as the relative increases, calculated as percentages of the initial control values which were taken as 100 %.

IL-1 activity was measured by a modified D10S assay as described by Orencole and Dinarello (1989) in which IL-1 was measured by testing the capacity of serum samples to stimulate proliferation of the IL-1 sensitive cell line, a subclone of the murine T-helper cell line D10.G.4.1. (kindly provided by C.A. Dinarello, Tufts Univ. Boston, Mass. USA). Results are given in femtograms per milliliter that were calculated from a standard curve obtained by using ultrapure IL-1 preparations.

IL-6 activity was measured by using the IL-6-dependent murine hybridoma cell line B9 (supplied by L.A.Aarden, Clinical Laboratory of Netherlands, Amsterdam) according to Shalaby *et al.* (1989). The specificity of IL-1 and IL-6-induced proliferation was confirmed as described earlier (Kataranovski *et al.* 1992), i.e. by neutralizing cell proliferation with polyclonal anti-human anti IL-1 antibody (Genzyme, Mass, USA), cross-reactive with rat IL-1, and by anti IL-6 antibody (kindly donated by Dr G. Bendixen, Univ. Hospital, Copenhagen, Denmark), cross-reactive with rat IL-6.

TNF activity was measured by using the L-929 fibroblast assay (Meager et al. 1989) in which cytotoxic activity of serum samples was detected in the presence of actinomycin D (Sigma Chemical Co., St. Louis, Mo, USA). Viable cells were stained with methylene blue, acid solubilized and their optical density determined with microplate spectrophotometer (Behring ELISA processor, Behringwerke AG Diagnostica). The specificity of TNF-induced cytotoxicity was confirmed by neutralizing anti-mouse TNFα antibody, cross-reactive with rat TNF (Pejnović *et al.* 1995).

The results are expressed as means ± S.E.M. from 6-8 animals Comparisons of the means between groups of experimental and sham-injured animals were performed by Mann-Whitney's test. The relationships between levels of cytokine bioactivity and concentrations of acute phase proteins in the serum were examined by regression analysis.

Table 1. Hematocrit and rectal temperature values following thermal injury

Time (hours)	Hematocrit (%)	Rectal temperature (°C)
1	0.600 ± 0.007**	34.4 ± 1.73**
3	0.667 ± 0.033**	32.5 ± 0.48**
6	0.597 ± 0.038**	34.7 ± 1.12**
12	0.470 ± 0.025	36.9 ± 0.97
24	0.478 ± 0.025	37.9 ± 0.50*
72	0.444 ± 0.028	36.8 ± 0.34
Initial control values		
	0.498 ± 0.021	36.8 ± 0.30

Significant difference vs control levels at $*p<0.05$, $**p<0.01$.

Results

A significant drop of rectal temperature was noted 1, 3 and 6 h after thermal injury compared to initial control values (Table 1). This initial decrease in temperature was followed by a significant rise on day 1 following thermal injury. Increased hematocrit values were measured 1, 3 and 6 h after trauma infliction reflecting hemoconcentration at the early postinjury period. No changes in temperature or hematocrit values were noted in sham-injured group of rats.

Serum IL-1 activity was increased throughout the examined post-injury period, with highest values in the first 3 h. Thereafter IL-1 activity was slowly decreasing, but was still higher than in sham-injured animals at the corresponding times (Fig. 1A). No significant changes in activity of this cytokine were noted in the sera of sham-injured animals at these periods.

No changes in the levels of serum TNF activity were noted up to 12 h following the scalding procedure, when a progressive rise of TNF activity was found in comparison to values at corresponding times in sham-injured animals (Fig. 1B). No changes of TNF activity were found in the sera of sham-injured animals up to days 1 and 3 following injury, when an increase was noted as compared to initial levels, i.e. levels detected in groups of non-manipulated animals.

Increased levels of serum IL-6 activity, in comparison to values detected in serum samples of sham-injured animals, were already found one hour after scalding, followed by a progressive rise of activity up to

day 1 which then decreased on day 3 after thermal injury (Fig. 1C). No changes of IL-6 activity in the sera of sham-injured animals compared to initial levels were noted up 12 h following injury.

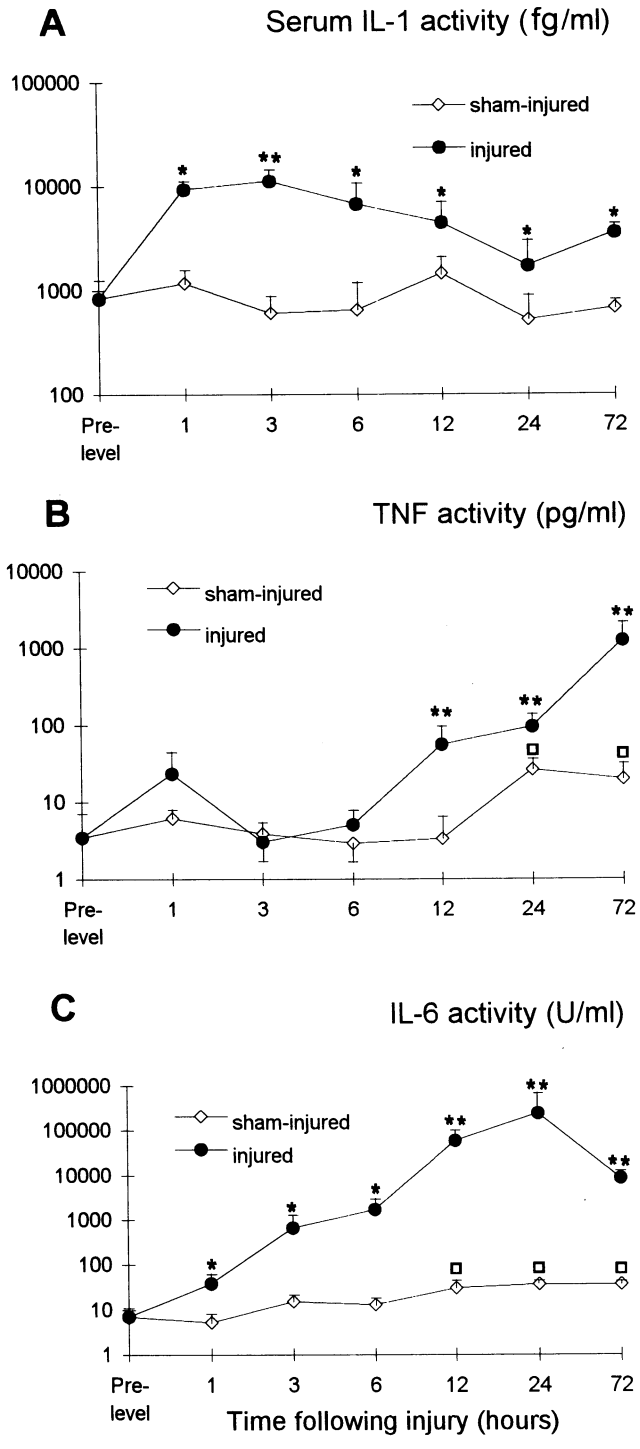

Fig. 1. *Time course of changes in serum cytokine activity following thermal injury. Significance at *p<0.05, **p<0.01 vs sham-injured rats. (□) significance vs control levels.*

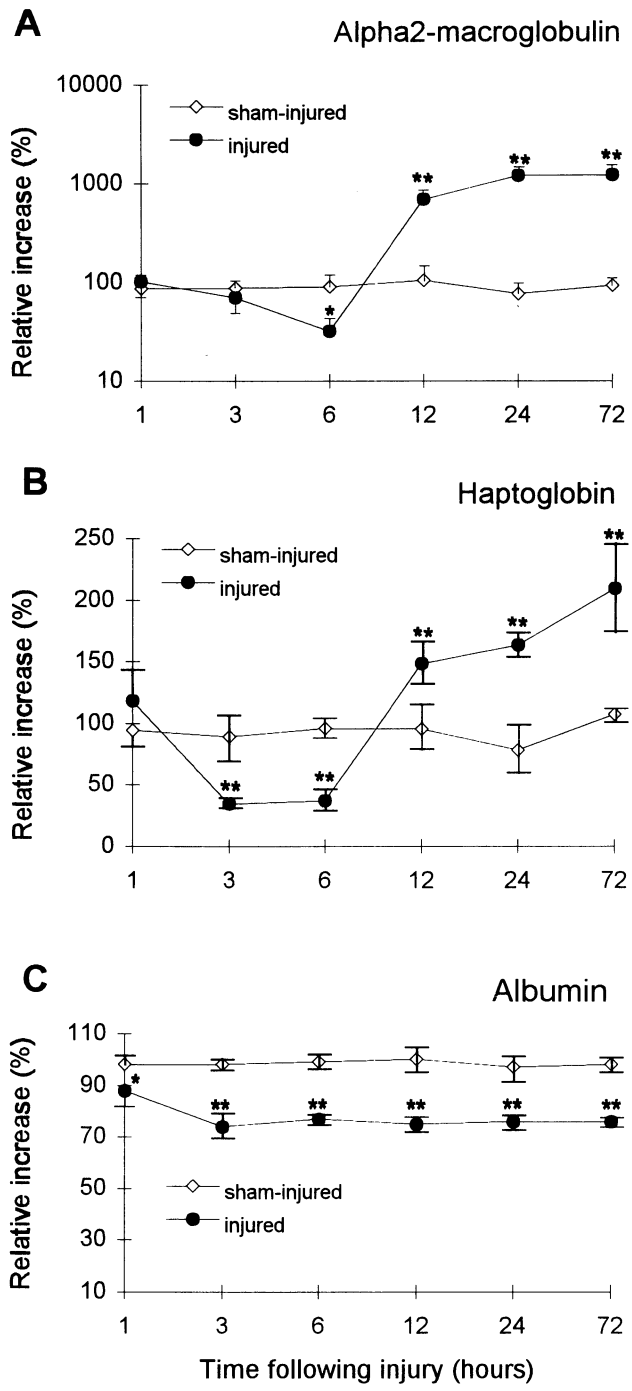

Fig. 2. *Changes in relative concentrations of the individual acute phase proteins in the sera of rats after thermal injury. Data are expressed as percentages of initial control values. Significance at *p<0.05, **p<0.01 vs sham-injured rats.*

The relative concentrations of alpha$_2$-macroglobulin and haptoglobin in the sera of thermally

injured rats were time-dependent. The levels of both these serum proteins, compared to their levels in the sera of sham-injured animals, were not changed one hour after injury. However, alpha$_2$-macroglobulin levels decreased at 6 h and haptoglogin levels 3 and 6 h after thermal injury. This initial decrease was followed by a dramatic increase in alpha$_2$-M and haptoglobin levels from 12 to 72 h following scalding (Figs 2A and 2B). No significant changes were noted in the sera of sham-injured animals during this time-period. In contrast to the increase of these acute phase proteins from 12 to 72 h following thermal injury, the levels of albumin were significantly decreased from the first hour after thermal injury in comparison to sham-injured animals (Fig. 2C).

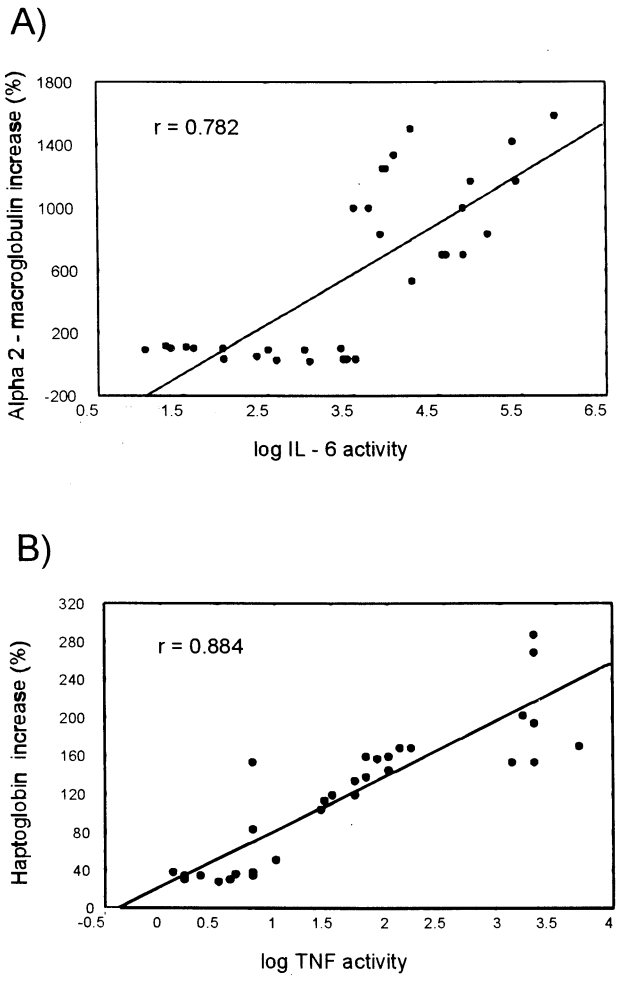

Fig. 3. *The regression line for changes in relative serum concentrations of alpha$_2$-macroglobulin and log IL-6 activity (A) and haptoglobin and log TNF activity (B).*

A significant correlation (r=0.782, p<0.001) was found between the time-dependent increase of serum IL-6 activity and relative serum levels of alpha$_2$-macroglobulin (Fig. 3A), and between changes in the levels of serum TNF activity and haptoglobin (r=0.884, p<0.001) (Fig. 3B). The respective regression formula for correlation between IL-6 activity and alpha$_2$-macroglobulin was y = –543.8 + log IL-6 activity*319.4, where *y* is relative concentration of alpha$_2$-macroglobulin. The corresponding regression formula for correlation between TNF and haptoglobin is y = 22.76 + log TNF activity*58.64, where *y* was relative concentration of haptoglobin.

Discussion

In the present report, the early cytokine response and acute phase protein response was studied in rats by using the experimental model of thermal injury. Full-thickness scalding of the skin triggered a systemic response exhibiting changes in body temperature and hematocrit. A significant early drop of body temperature as well as hemoconcentration following thermal injury reflect hypothermic and hypovolemic aspects of an early "hypometabolic" phase of generalized reaction to injury (Zweifach 1986). The rise in body temperature observed 24 h following scald infliction, probably reflects a "pyrogenic effect" of inflammatory cytokines demonstrated in experimental models of inflammation (Rothwell 1990, Caldwell *et al.* 1997) and suggested in thermally injured patients (Childs *et al.* 1990).

Thermal injury in rats triggered an early systemic cytokine response as revealed by time-dependent changes in the levels of IL-1 and IL-6 activity in the serum (Fig. 1). There are no available data in the literature regarding early circulating IL-1 levels in burn injury. Only a borderline increase of IL-1-β (a circulating form of IL-1) was noted in the plasma of burned patients for several days following injury in comparison to initial levels at the time of admission (Cannon *et al.* 1992). However, these values were not significantly elevated when compared to age- and sex-matched controls (Wogensen *et al.* 1993). The discrepancies between these data were explained by the presence of a natural inhibitor of IL-1 activity, interleukin-1 receptor antagonist (IL-1 RA), the levels of which are enhanced in thermal injury (Mandrup-Poulsen *et al.* 1995). Our data on the decrease of IL-1 activity, detected at later times following scalding, might also reflect the presence of inhibitory activity for IL-1, which could have masked the activity of this cytokine.

The immediate rise of IL-1 activity detected in

our study might reflect early induction of this cytokine in various organs as reported recently in rats with full-thickness thermal injury to the skin (Mester *et al.* 1994). A triggering role of interleukin-1 in the cytokine network was suggested in the above study, highlighting the possible function of this cytokine in establishing the inflammatory response associated with thermal injury. The rise of IL-1 activity, which preceded that of TNF and IL-6 in our study, is in general agreement with the data from other models of inflammation showing that inflammation-related cytokines work in sequence (Cerami 1992, Baumann and Gauldie 1994) and that IL-1 is the first cytokine to appear in systemic circulation in the inflammatory cascade.

Although some elevation of TNF and IL-6 activity in the sera of sham-injured animals were noted on days 1 and 3 compared to initial levels, these could not, at least on day 3 for TNF and on both days for IL-6, contribute significantly to the rather high values of serum cytokine activity detected at these time points in the sera of thermally injured animals. Increased values of these cytokines in the sera of sham-injured rats probably reflect the effect of ether anesthesia. The increased activity of TNF and IL-6 detected 1 and 3 days after thermal injury is in agreement with the available data from thermally injured patients (Guo *et al.* 1990, Endo *et al.* 1993) in which elevated levels of these cytokines were noted at the same time-points. Although the rise of TNF in some patients coincided with the presence of endotoxin, no correlation was found between maximum plasma TNF and endotoxin levels (Endo *et al.* 1993). Low values of TNF activity detected in the early period (1 to 6 h) following scald infliction, might reflect either the absence of TNF activity or the presence of TNF inhibitory activity. Data from various kinds of trauma indicated increased values of soluble TNF receptors, cleaved fragments of the extracellular portion of TNF receptor which are competitive inhibitors for TNF (Tan *et al.* 1993).

The pattern of changes of IL-6 activity (early rise and progressive increase) in the circulation during the initial period following thermal injury is in concordance with the qualification of IL-6 as an "alarm cytokine" (Nijsten *et al.* 1991), a reliable marker of the acute inflammatory response (Ohzato *et al.* 1993) with an essential role in the early mobilization of the metabolic response under the conditions of injury. The increased levels of IL-6 activity early after thermal injury demonstrated in the present study complement the data from studies in thermally injured patients in which permanently elevated levels starting from day 1 postburn were found (Guo *et al.* 1990, Schluter *et al.* 1991), providing more complete pattern of serum IL-6 kinetics in thermal injury.

Significant correlations between the time-course of changes in IL-6 activity and alpha$_2$-macroglobulin levels, and between TNF activity and haptoglobin levels, suggest a relationship between changes in cytokine levels and AP synthesis in the inflammatory response of thermally injured rats. Our results are in accordance with the data which had demonstrated positive correlation between IL-6 and C-reactive protein, CRP, a major human acute phase protein in thermally injured patients (Nijsten *et al.* 1987). As IL-6 preceded the appearance of CRP, it was suggested that this cytokine is a principal mediator of the acute phase response in these patients. On the basis of the observed kinetics of IL-6 activity in thermally injured rats and the experimental data, which directly demonstrated the important role of administered IL-6 in the induction of alpha$_2$-macroglobulin and haptoglobin in rats *in vivo* (Geiger *et al.* 1988), we believe that IL-6 is a major mediator of thermal injury associated with the acute phase response in rats. The increase in serum levels of acute phase proteins observed in this study might result from their increased synthesis in the liver, which is preceded by increased transcription of genes for alpha$_2$-macroglobulin and haptoglobin (Ševaljević *et al.* 1988). A decrease in the concentration of alpha$_2$-macroglobulin and haptoglobin AP proteins demonstrated 3 and 6 h following injury does not seems to be influenced by a reduction in plasma volume, indicated by hemoconcentration at these time-points (Table 1), because the levels of alpha$_2$-macroglobulin and haptoglobin were not altered one hour after scalding, when increased hematocrit values were also demonstrated. Serum levels of AP proteins reflect a balance between their synthesis and utilization and/or clearance so that the observed decrease might be a consequence of changes in either of these processes. As the thermally injured skin is a source of large quantities of lysosomal enzymes, increased utilization of alpha$_2$-macroglobulin and haptoglobin, serum and tissue anti-proteases, respectively (Baumann and Gauldie 1994), could contribute to their reduced serum levels. Decreased serum albumin levels could be due to decreased synthesis of hepatic mRNA for albumin (Ševaljević *et al.* 1988) and due to accelerated albumin clearance from circulation (Davies 1976).

Studies of the systemic cytokine response were mostly conducted in the context of endotoxemia and sepsis (Marano *et al* 1990, Drost *et al.* 1993). These studies implicated the involvement of bacterial endotoxins in development of the cytokine response to thermal injury and have ascribed a role to IL-6 and TNF in the response to thermal injury-associated infection. Circulating bacterial endotoxin might arise from the wound infection in the so-called "infection stage" which follow the initial "shock stage" period after thermal injury (Evans 1969) or from the gut by transmural migration of viable indigenous gastrointestinal tract microorganisms, a process called "bacterial translocation" (Alexander *et al.* 1990). The cytokine response to thermal injury in our study does not seems to be the result of products of microbial origin, because negative results were obtained using *Limulus amebocyte* lysate assay for endotoxin in serum samples obtained during the 3-day period following thermal injury. Moreover, positive indigenous bacterial translocation, a major source of endogenous endotoxins, was not detected after burn injury of 20% body surface in rats (the area covered with thermal injury in our model), but only under the conditions of more severe thermal injury, covering 40 % body surface in rats (Maejima *et al.* 1984). The rise of cytokines in our study might rather be an integral part of the inflammatory response to tissue injury induced by full-thickness scalding of the skin. A number of mechanisms could contribute to this biological response, including classical pathways of inflammation as well as stress hormones. It is proposed that thermal injury of the skin results in a local release of inflammatory mediators which might initiate the systemic inflammatory cascade (Rodriguez *et al.* 1993). Experimental evidence documents the existence of local cytokine response after skin injury. The thermally injured area is a rich source of IL-1 and IL-6 in both rats (Pejnović *et al.* 1995, Kataranovski *et al.* 1996) and humans (Ono *et al.* 1995). The neutrophil chemoattractant released from thermally injured skin (Garner *et al.* 1994) might mediate neutrophil chemotaxis into the injured area. Subsequent neutrophil activation and degranulation could trigger local inflammatory pathways with oxidants and products of arachidonic acid metabolism, known stimulators of cytokines (Kasama *et al.* 1989) and thus initiate the cytokine response. Other potential sources of cytokines in thermal injury include phagocytic cells (Schluter *et al.* 1991), lymph nodes (Kataranovski *et al.* 1992, Ohzato *et al.* 1993), hepatocytes (Ohzato *et al.* 1993) and uninjured skin (Kataranovski *et al.* 1996, Kawakami *et al.* 1997).

The stress of thermal injury could significantly contribute to the cytokine response and might include effects of stress hormones including corticosteroids the concentrations of which are raised in the rat after thermal injury (Mortensen *et al.* 1972). A relationship might exist between catecholamines and IL-6 (van Gool *et al.* 1990) and between the corticotropin-releasing factor (CRF) and IL-6 (Leu and Singh 1992). Both these stress factors have been shown to stimulate IL-6 production.

Dynamic changes in the circulating levels of IL-1, TNF and IL-6 implicate the role for these cytokines in the early response to thermal injury in rats. Cytokines probably mediate the process of inflammation by raising body temperature and acute phase proteins. Both effects are regarded as beneficial to the host as regulators of physiological equillibrium (Childs *et al.* 1990, Weissman 1990). In addition, the thermal injury-induced rise of cytokines might positively modulate defense mechanisms as was demonstrated for components of the humoral immune response in clinical studies (Nijsten *et al.* 1991). Inflammatory cytokines produced early in the postburn period might prime leukocytes to respond one week later to endotoxin by further production of these cytokines as was demonstrated in thermally injured rats (Wu *et al.* 1995). Increased levels of cytokines could lead to hypermetabolism associated with an exaggerated inflammatory response, seen clinically after the first postburn week (Youn *et al.* 1992).

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