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# Monitoring of elastase in plasma of burned patients in relation to other inflammation parameters

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*Twenty burned patients divided in three groups according to the severity of the lesions were investigated at 1- or 2-day intervals for up to 5 weeks after injury. Plasma elastase levels were elevated during the first day after injury and were correlated with the area of the burns. However, plasma elastase was rapidly bound and inactivated by protease inhibitors. Leucocyte counts, fever and the concentration of α-1-proteinase inhibitor were not correlated with the extent of the burn. The rise of plasma elastase was not accompanied by consumption of the elastase inhibitory capacity (EIC) of plasma, which increased to a plateau around day 5. The EIC values were in accord with the rise of α-1-proteinase inhibitor, the major anti-elastase agent in plasma. Studies of blister fluid in eight patients showed that the elastase content was higher than that of corresponding plasma, while the concentration of α1-proteinase inhibitor and the EIC were comparable with those of plasma. Measurements of the levels of tumour necrosis factor released by stimulated macrophages in five patients with major burns showed no significant increase compared with controls.*

## Introduction

Burn injuries of considerable extent modify many physiological and biochemical functions that involve vascular permeability, coagulation and complement activation, leucocytosis, increases in acute phase proteins and several neuroendocrine responses. (Daniels et al., 1974; Faymonville et al., 1987; Travis and Salvesen, 1983). Although these events are linked to the inflammatory reaction that regulates the biological defence functions and triggers the healing process, it is well known that the same mechanisms may cause pathological effects such as tissue destruction, pulmonary leucostasis, intravascular coagulation, shock and even immunosuppression with consequent increased susceptibility to infections (Alexander, 1967; Ozkan et al., 1988; Bjornson et al., 1989; Weiss, 1989). Among the various local and systemic reactions that follow burn injury, one of the major factors that determines outcome is the activation of neutrophils that are massively recruited from marginated and marrow pools. These cells migrate from the blood into the burned areas where they phagocytose microbial invaders and release proteolytic enzymes and toxic oxygen derivatives. An understanding of the role played by the various humoral and cellular events that regulate the balance between the positive and negative effects of inflammation is required for a rational therapeutic approach (i.e. the administration of anti-inflammatory agents or of antiproteolytic drugs) (Stratta et al., 1983; Barisoni et al., 1987).

These questions may be addressed by looking for clinical and laboratory indices that evaluate the burn-induced inflammatory responses.

This report monitors neutrophil elastase activity in the plasma of patients with different burn scores, as compared with leucocyte counts and antiprotease concentrations.

Anti-elastase activity of plasma is mainly accounted for by α-1-proteinase inhibitor (Weiss, 1989), a glycoprotein that rapidly and irreversibly inhibits neutrophil elastase by forming an enzyme–inhibitor complex. However, quantification of this protein may not reflect the actual inhibitory capacity of plasma, because this inhibitor may be inactivated by oxidative mechanisms (Travis and Salvesen, 1983; Weiss, 1989) which are likely to occur during acute burn-induced inflammatory responses.

In order to evaluate the balance of elastase and anti-elastase mechanisms in burn plasma we measured both the concentration of α-1-proteinase inhibitor and the inhibitory activity of plasma. This latter parameter is termed elastase inhibitory capacity (EIC).

## Materials and methods

Blood samples (10–20 ml) anticoagulated with citrate were obtained from 20 burned patients with different burn severities (calculated as units of burned skin (UBS) = total burn + 3 × per cent full skin thickness burn) and healthy controls. Samples were immediately transferred to an ice-bath, then centrifugated at 4°C for 15 min (600 × ***g***). One millilitre aliquots of plasma were frozen in liquid nitrogen and then stored at −70°C. Once thawed, samples showing macroscopic precipitates were clarified by centrifugation for 2 min in an Eppendorf microfuge.

Samples of blister fluid were obtained from eight patients with UBS > 100, aliquoted and stored as described for the plasma samples. Elastase was determined by enzyme-linked


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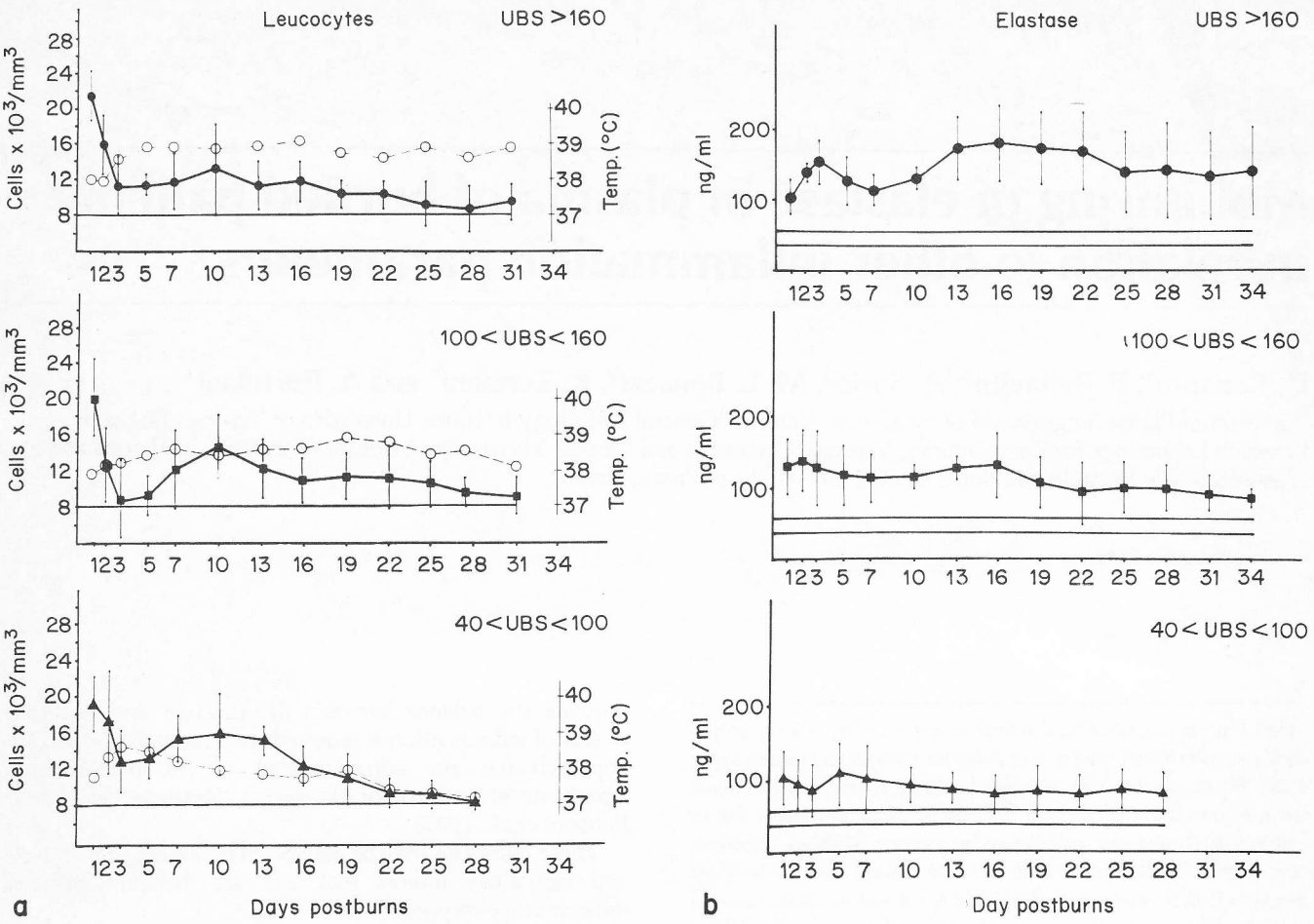



**Figure 1.** Total leucocyte count (●, ■, ▲) and body temperature (○) (a), plasma elastase (b), elastase inhibitory capacity (c), α-1-proteinase inhibitor (d) of burned patients, divided into three groups according to the severity of the injury. The group with UBS > 160 was composed of seven patients, two of them died (one on day 46, the other on day 35); the group with UBS 160–100 was composed of

immunosorbent assay (Merck), which quantitates the complex elastase α-1-proteinase inhibitor (Plow, 1982; Neumann et al., 1984; Beutler and Cerami, 1985; Speer et al., 1987).

Elastase activity was determined by using the cleavage of succinyl alanyl-alanyl-alanyl-*p*-nitroanilide (Merritt et al., 1983; Ozkan et al., 1988). One millilitre of assay medium (0.3 mM substrate in 0.2 M Tris-HCl, pH 8.0, containing 1 mg/ml of bovine serum albumin) was added to 0.15 ml plasma or to porcine pancreatic elastase standard (Sigma) and incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.2 ml of 2 M acetic acid and the absorbance versus a blank was measured at 410 nm.

EIC was evaluated by mixing plasma samples (2–7 µl) with 10 µg of porcine pancreatic elastase at room temperature for 30 min in 100 µl of 0.2 M Tris-HCl, pH 8.0. Then 1 ml of the substrate for elastase, in the same medium reported above, was added and the reaction proceeded for 30 min before being blocked and measured as described above. Preliminary experiments showed that the reaction rate was linearly related to the amount of added plasma when the inhibition was between 20 and 80 per cent of control values measured without plasma. For this reason, the plasma to be added was carefully titrated in each experiment. EIC was calculated as percentage inhibition with respect to the control reaction and the results were expressed as units EIC/ml of plasma, where 1 unit is represented by 50 per cent inhibition of the activity of 10 µg of porcine elastase.

α-1-proteinase inhibitor was determined by nephelometry using an Automatic Immunochemical System (AutoICS Beckman Instruments Inc., Fullerton, CA, USA) with the specific reagents commercially provided by Beckman (Baldwin et al., 1979).

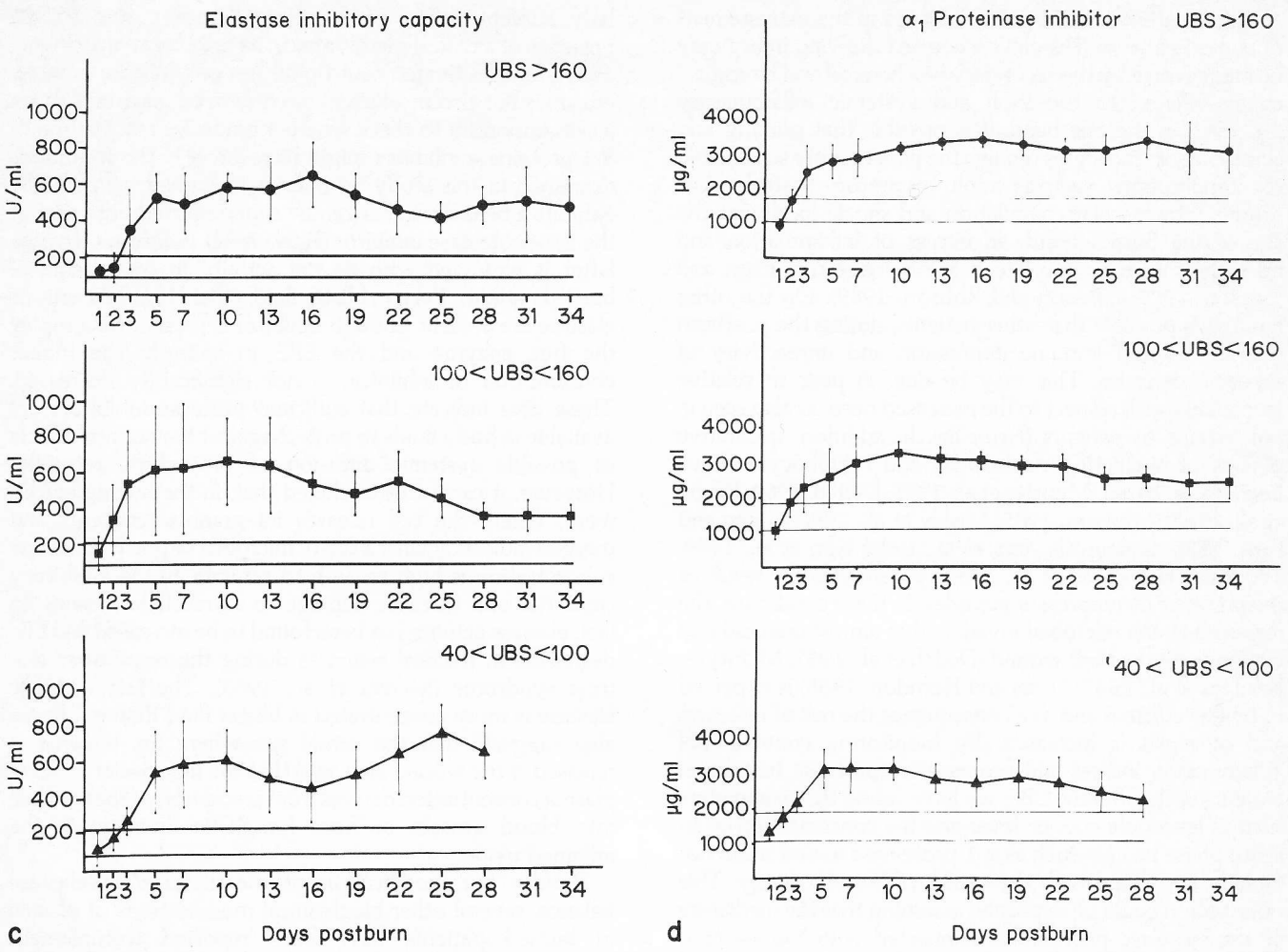
Tumour necrosis factor alpha was determined by enzyme-linked immunosorbent assay (Biokine-TNF) (Mest et al., 1986; Scuderi et al., 1986; Cuturi et al., 1987).

## Results

Leucocyte count, plasma elastase, α-1-proteinase inhibitor and EIC were measured in 25 burned patients at 1- to 2-day intervals over a 5-week period after injury (*Figure 1*). The patients have been divided into three groups according to the severity of the disease, measured as UBS: group A, UBS > 160; group B, UBS 160–100; group C, UBS 100–40.

Leucocyte counts (*Figure 1a*) showed a marked peak during the first and second days postburn, then a progressive decrease until day 3. Subsequent values were close to the expected normal range, apart from leucocyte peaks which were associated with fever in almost all the patients. This fever was secondary to inflammatory reactions and possibly to wound infection, common complications in these patients.

During both the primary and secondary leucocytosis, over 80 per cent of the leucocytes were neutrophils. The pattern of leucocyte behaviour was similar in all the three groups, irrespective of the severity of injury. Possibly, a




seven patients; the group with UBS 100–40 was composed of six patients, four of whom recovered before day 35 and were discharged from hospital. Body temperature is reported as the mean maximal temperature values.

slightly higher secondary leucocytosis was present in the patients with UBS 100–40. Plasma elastase concentrations (*Figure 1b*) were markedly elevated in all the patients, but showed net differences in the three groups. In the most severely affected group, the enzyme levels increased during the first 2–3 days, then slightly decreased afterwards. They showed a further marked increase around 10–16 days, with a broad peak that lasted for 15–20 days. In the less severely injured group (UBS 100–40) the second peak was much smaller and short-lasting. In the group with UBS 100–160 the observed plasma elastase concentrations were intermediate between the other two groups. As shown in *Figure 2*, plasma elastase activity and UBS were statistically correlated, with $r = 0.63$ and $P < 0.01$. Since it is known that elastase in plasma is rapidly bound and inactivated by proteinase inhibitors, it was of interest to evaluate whether the rise of plasma elastase was accompanied by the consumption of the EIC of plasma. As shown in *Figure 1c*, the plasma EIC did not decrease after burning injury, but progressively increased with a plateau around day 5. This pattern was similar in the three groups of patients, indicating that the increase in EIC was maximally triggered even by burns with UBS 100–40, and was largely in excess with respect to the amount of inhibitor that was complexed to elastase. In fact the free elastase activity was almost undetectable in all the tested plasma samples (data not shown).

The data of EIC are in accord with the results shown in *Figure 1d*, where α-1-proteinase inhibitor, the major anti-elastase agent of plasma (Weiss, 1989), was significantly elevated in all the burned patients, and its pattern was practically superimposable on that of EIC. Neutrophil elastase can solubilize elastin but also fibronectin, collagen and proteoglycan (Roughly and Barret, 1977; McDonald and Kelly, 1980; Faymonville et al., 1987). In burned patients it may be involved in the degenerative processes that locally affect the dermal tissues. In order to verify whether elastase was released at the site of burn injury, blister fluid was collected from eight patients and analysed (*Table I*).

A possible mediator of the activation of leucocytes and of the adherence of these cells to endothelia is tumour necrosis factor (TNF) (Gamble et al., 1985; Berger et al., 1988). The presence of this cytokine in the plasma of burned patients could be implied by the intense inflammatory process in the wound that could stimulate the production of macrophage-derived cytokines, including TNF. However the search for TNF in the plasma in five patients with UBS > 160 was unsuccessful (*Figure 3*), indicating either that this cytokine is not released in the circulation in this condition, or that it is rapidly utilized so that its concentration does not increase.

## Discussion

The present study was focused on some of the major modifications that occur in the blood of patients in the postburn period. Correlating clinical evolution and bio-

chemical patterns could potentially aid in the management of burned patients. The clinical course following injury may be made worse by the secondary biochemical and biological events related to the local and systemic inflammatory process. On the one hand, it is possible that priming and activation of leucocytes renders the patient more susceptible to complications such as adult respiratory distress syndrome, intravascular coagulation and shock; locally, at the site of the burn wound, an excess of inflammation and microthrombi may enhance tissue damage (Arturson and Jonsson, 1979; Eriksson and Robson, 1989). On the other hand, it is possible that other patients, during the postburn course, develop immune depression and unreactivity of aspecific defences. This may be due, in part, to relative leucopenia with respect to the increased need, as also seen in our groups of patients (*Figure 1a*). In addition, qualitative defects of circulating neutrophils and lymphocytes have been documented (Munster et al., 1973; Deitch, 1984; Wood et al., 1984; Arturson, 1985; Mistry et al., 1986; Green and Faist, 1988; Guang-Xia Xiao et al., 1988; Kim et al., 1989; Teodorczyck-Injeyan et al., 1989), possibly as the result of the presence of suppressor peptides. In these conditions, the response to the microbial invaders that almost unavoidably contaminate the burn wound (Deitch et al., 1987; McIntyre-Brydges et al., 1987; Desai and Herndon, 1988) is expected to be less efficient and as a consequence the risk of infection and of sepsis is increased. By monitoring conventional inflammation indices and protease/antiprotease balance in patients with different UBS, we have found that parameters such as leucocyte counts, fever and the concentration of an acute-phase protein such as α-1-proteinase inhibitor are not directly correlated with the extent of burning injury. This observation could be explained assuming that the mediators of the systemic phenomena connected with trauma (and possibly with infection in the second period of the clinical course) trigger the maximum response in target systems (bone marrow, liver, neuroendocrine system, etc.) even in relatively minor injuries. Moreover, it is possible that in severely injured patients, leucocytosis and increased hepatic protein synthesis are counter balanced by the presence of inhibitors (Ozkan et al., 1988), cardiovascular failure and shock, or, as in the case of neutrophils, adhesion to activated endothelia and chemotactically driven extravasation. Others have observed that the acute phase response in burned patients is quite independent of the severity of burns (Faymonville et al., 1987).

This work clearly demonstrated that among the considered blood parameters, the most pertinent index that may be correlated with the severity of injury is the concentration of neutrophil elastase, as assessed by the enzyme-linked immunoassay for the complex enzyme α-1-proteinase inhibitor (Stratta et al., 1986; Ozkan et al., 1988). The less severely injured patients have less elastase and healing is accompanied by disappearance of the enzyme complex from plasma (*Figure 1b*).

The rise of elastase-inhibitor complex in plasma is not accompanied by a disruption of the balance between proteases and antiproteases and by the appearance of free elastase in plasma. This fact is due to the speed of formation of the complex (Travis and Salvesen, 1983)) and to the overwhelming amount of inhibitor with respect to elastase. From the data of *Figure 1* it can be seen that in terms of weight, the complex elastase/inhibitor reaches maximal values of 200 ng/ml, thus representing at the most 1/5000 of the inhibitor (range: 1–4 μg/ml).

It has been shown that α-1-proteinase inhibitor is particularly susceptible to oxidative inactivation, due to the presence of a critical methionine in its reactive centre (Weiss, 1989). Since activated neutrophils not only release granular enzymes but also produce oxygen-derived reactive species, it was important to check whether oxidative inactivation of α-1-proteinase inhibitor might have a role in the conditions examined in this study. In plasma of burned patients EIC exhibits a behaviour that can be superimposed onto that of the α-1-proteinase inhibitor (*Figure 1c–d*), indicating that the latter is endowed with its full activity in the plasma of burned patients. Even in blister fluid, where high amounts of elastase are present, enough inhibitor is present to complex the free enzyme and the EIC, in spite of the higher consumption of inhibitor, is not significantly decreased. These data indicate that sufficient protease inhibitors are available in body fluids to protect against the harmful effects of possible systemic diffusion of proteolytic activities. However, it cannot be excluded that, in the microenvironment, where the cell releases its granular contents and oxygen radicals against a cell of microbial target, the elastase concentration is high enough to saturate all the inhibitory capacities and to cause damage to extracellular tissues. In fact, elastase activity has been found to be increased and EIC decreased in tracheal aspirates during the respiratory distress syndrome (Merritt et al., 1983). The fact that the elastase is more concentrated in blister fluid than in plasma also suggests that the actual site where the enzyme is released is the wound area, and that the increase of elastase plasma concentration derives from resorption of the exudate into blood vessels, or from lymphatic drainage of the inflamed tissue.

Besides the modification of the elastase/anti-elastase balance, several other biochemical modifications of plasma in burned patients have been reported (complement activation, C-reactive protein increase, etc.). However, little is known of the possible participation of cytokines that regulate many of the biological events of inflammation. We have tried to determine the concentration of TNF, one of the most powerful cytokines produced by activated macrophages, which has been suggested to play a major role in acute systemic phenomena such as shock (Beutler et al., 1985; Tracey et al., 1986; Oliff et al., 1987; Waage et al., 1987). However, TNF was practically undetectable in the plasma of our patients, an observation that rules out the possibility of utilizing the intravascular concentration of this cytokine as a parameter for following up these patients and for predicting possible complications. It should be pointed out that the immunoassay that was utilized in this study is designed for detecting only the active form of the molecule and that therefore our data do not exclude that in vivo, after burn injury, TNF is actually produced and rapidly metabolized or taken up by cell receptors.

The data reported here suggest that the optimal monitoring of inflammation in burned patients should take into consideration the evaluation of plasma elastase as a marker of leucocyte activation, while other conventional parameters are of less help for this purpose. More work is necessary to define if the measurement of elastase could be utilized for predicting the appearance of clinical complications or for choosing a rational therapeutic approach, based, for example, on the use of anti-inflammatory agents (where there is risk of ARDS or shock) or of antibiotics and immunostimulating agents (where immunosuppression is the major problem). The data showing the presence of an efficient endogenous system for protection against leucocyte elastase activity suggests that in most patients there is

**Table I.** Elastase and elastase inhibitor in plasma (PL) and blister fluid (BF) of eight patients (UBS > 100) during day 1 postburn

| | *Elastase (ng/ml)* | | *Total elastase inhibitory activity (U/ml)* | | *α-1-proteinase inhibitor (μg/ml)* | |
|---|---|---|---|---|---|---|
| *Patient* | *PL* | *BF* | *PL* | *BF* | *PL* | *BF* |
| 1 | 58.1 | 404.4 | 238 | 118.7 | 1300 | ND |
| 2 | 165.2 | 864.4 | 232.5 | 207.2 | 1045 | 1010 |
| 3 | 78.8 | 331.2 | 163 | 144.5 | 884 | 848 |
| 4 | ND | 77.8 | 222.7 | 236.7 | 1070 | 1030 |
| 5 | 151.5 | 260.8 | 91.3 | 116.1 | 1060 | 928 |
| 6 | 130.3 | 315.4 | 119 | 126.6 | 1100 | 1130 |
| 7 | 77.7 | 214.6 | ND | ND | 926 | 1010 |
| 8 | 89.4 | 394.6 | ND | ND | ND | 1140 |
| Control | 49.8 ± 10 | | 143.6 ± 61 | | 1386 ± 359 | |

ND, not detected.

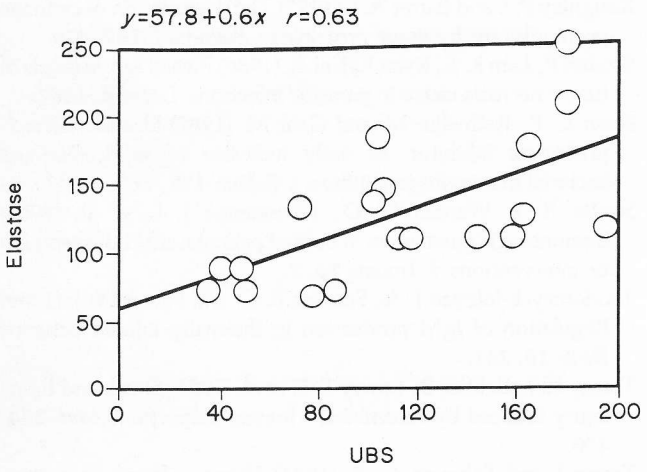



**Figure 2.** Correlation between plasma elastase and UBS, and between blood leucocyte count and UBS in burned patients.

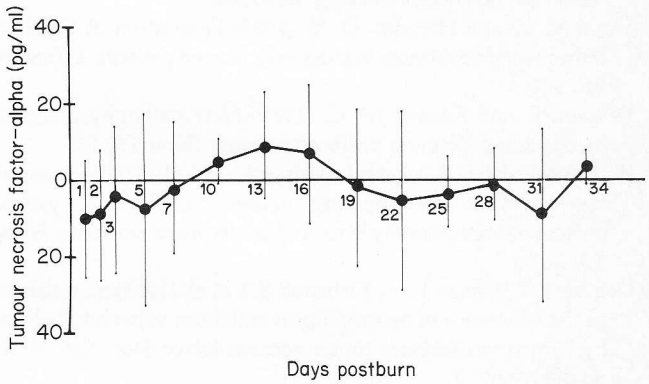



**Figure 3.** Tumour necrosis factor concentration in a group of severely burned patients.

no need for the additional administration of antiproteolytic drugs. However, it cannot be excluded that in rare individual cases, especially in the presence of concomitant hepatic failure, the amount and the activity of proteinase inhibitors could become insufficient.

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