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Leukotoxin, a linoleate epoxide: Its implication in the late death of patients with extensive burns

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Abstract

Burn death based on circulatory shock is often encountered after recovery from primary shock in patients with deep and extensive burns, *i.e.,* late death. Several toxic substances have been proposed, however, the responsible substance remains obscure. Since we have found leukotoxin, a highly cytotoxic linoleate epoxide biosynthesized by neutrophils, in the burned skin, in the present study we determined plasma leukotoxin concentrations in various degree of 30 burn patients. C-reactive protein and circulatory white blood cells were also measured. A significantly high mortality rate of patients with extensive burns (burn surface area over 70%) was observed compared with that in patients with burn surface area under 70%, and significantly high leukotoxin concentrations were observed within a week, and 3 weeks after the thermal injury in patients with extensive burns compared with those in patients with burn surface area under 70%. There were two peaks of plasma leukotoxin concentrations, *i.e.,* the early phase (within 1 week) and the late phase (over 1 week) in patients with extensive burns. Plasma leukotoxin concentrations significantly correlated with burn surface area in the early phase, and similar correlations were observed in the late phase. A significantly high mortality rate (61%) of patients with peak leukotoxin concentrations over 30 nmol/ml was observed compared with 8% for those below 30 nmol/ml. Plasma leukotoxin concentration correlated significantly to C-reactive protein concentration, log (leukotoxin nmol/ml) = 0.042 × C-reactive protein (mg/dl) + 0.74, ($r = 0.83$, $P < 0.01$) in the late phase. From these results, it is concluded that leukotoxin is produced in patients with burns particularly in the late phase of extensive burns, and leukotoxin might play an important role in the tissue destructive procedure associated with severe burns. (Mol Cell Biochem **139:** 141–148, 1994)

Key words: extensive burns, late death, neutrophils, linoleate epoxide, leukotoxin

Introduction

Extensive burn is a life threatening condition, and hypovolemic shock, cardiac failure, renal failure and respiratory failure are major causes of burn death in the early phase. Furthermore, burn death based on circulatory shock is often encountered after recovery from primary shock in patients with deep and extensive burns, *i.e.,* late death, over 1 week after thermal injury. To explain the cause of late death, the existence of non-bacterial toxic substances has been proposed by various investigators, which could be produced in the lesions, transferred into the general circulation and cause multi-organ failure [1–3]. Nevertheless, the responsible toxin as a pure chemical substance remains obscure. In the 1980's, we reported that a heat-stable, ether soluble, *i.e.,* lipid like substance was extracted from burned skin [4, 5], and we demonstrated that this substance is a linoleate epoxide, 9,10-epoxy-12-octadecenoate, which is biosynthesized by neutrophils [6, 7]. We also found that leukotoxin existed in the plasma of two burn patients [8]. From our reports, leukotoxin was revealed to relax endothelin 1-induced pulmonary artery contraction [9] and radioimmunoassay for leukotoxin has

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been developed [10]. The hydroxyl radical produced by respiratory burst oxidase of neutrophil membranes was demonstrated to be a mediator of its biosynthesis [11]. This epoxide shows a highly toxic effect on cell function [12], and it was named leukotoxin. In previous reports, we detected leukotoxin in patients with severe inflammation such as adult respiratory distress syndrome (ARDS) and infectious endocarditis [13, 14]. Leukotoxin induces cardiac failure and disseminated intravascular coagulation in dogs [15, 16], which are characteristic complaints in patients with severe burns.

It is well known that burn surface area is a determining factor in the prognosis of burn patients [17]. Accordingly, in the present study, to clarify whether or not plasma leukotoxin concentration correlates with burn surface area and whether or not it correlates with the mortality of burn patients, we determined plasma leukotoxin concentrations of various degrees in 30 burn patients.

Materials and methods

Investigations were carried out in thirty patients with burns (over 30% of body surface area), between the ages of 8 and 80 yrs, admitted to the intensive burn care unit of Chukyo Hospital (Table 1). Plasma were collected and stored at –80° C until use. For the control, plasma were also collected from eight normal volunteers. Leukotoxin was extracted according to the method of Itaya and Ui [18]; 0.3 ml of plasma were mixed with 1 ml of 0.033 mol/l phosphate buffer (pH 6.4) and 6 ml of chloroform, and the mixture was shaken vigorously for 90 s. After centrifugation (1,200 × g, 5 min), 4 ml of chloroform was placed in another tube. The solution was evaporated on a rotary evaporator under reduced pressure. The samples were redissolved with 0.6 ml of methylalcohol.

Leukotoxin extracted was analyzed by high performance liquid chromatography (HPLC) as reported previously [7]. Briefly, the samples were injected into a Develosil-ODS column (5 μm particles, 0.46 ×15 cm and 0.46 × 25 cm; Nomura Chemical Co., Seto, Japan) mounted in a Shimadzu HPLC apparatus (Shimadzu Co., Kyoto, Japan). The solvent system used was a mixture of acetonitrile:water: phosphoric acid (73:27:0.1). The column oven (Shimadzu, CTO-6A) temperature was set at 30° C. Leukotoxin was detected by absorbance at 192 nm using an available wavelength detector (Shimadzu, SPD-6A), and its elution pattern was traced by a recorder (Shimadzu, C-R3A). To determine the chemical structure of leukotoxin, gas chromatography/mass spectrometry apparatus, Shimadzu GCMS-9020DF and 500 MHz ^{1}H NMR apparatus (JEOL GX-500, JEOL Co., Tokyo, Japan) were used as reported previously [6].

C-reactive protein in plasma was determined by the method of turbidimetric imunoassay, and circulatory red and white blood cells were measured by autoanalyser.

Reagents were purchased from Wako Chemical Co. (Tokyo, Japan).

Table 1. Clinical features of patients

No.	Age (yr)	Sex	Burn surface area (%)	Outcomes
1	17	M	30	survived
2	27	M	30	survived
3	40	M	30	survived
4	30	M	30	survived
5	26	M	30	survived
6	43	M	35	survived
7	57	F	35	survived
8	43	M	40	survived
9	42	F	40	died day 8
10	57	M	48	died day 18
11	70	M	50	survived
12	44	M	50	survived
13	76	F	50	died day 4
14	47	M	50	survived
15	44	M	60	survived
16	38	F	60	survived
17	49	M	60	survived
18	72	F	65	died day 35
19	43	M	70	died day 24
20	49	M	70	survived
21	80	F	70	died day 9
22	55	F	75	died day 51
23	20	M	80	survived
24	50	F	80	died day 17
25	17	F	80	survived
26	18	F	85	survived
27	53	M	85	died day 4
28	23	F	90	died day 84
29	8	F	92	died day 18
30	37	F	97	died day 4

Analysis of data

Analysis of variance with Bonferroni's test was used for comparison of leukotoxin concentration in patients with burn surface area over 70% and in those with under 70% [19]. Correlation coefficient analysis was based on Pearson's method [20]. χ^2-test was used for mortality. Probability (P) values of less than 0.05 were considered statistically significant. All values are presented as a mean ± standard error (S.E.).

This study was performed according to the declaration of Helsinki.

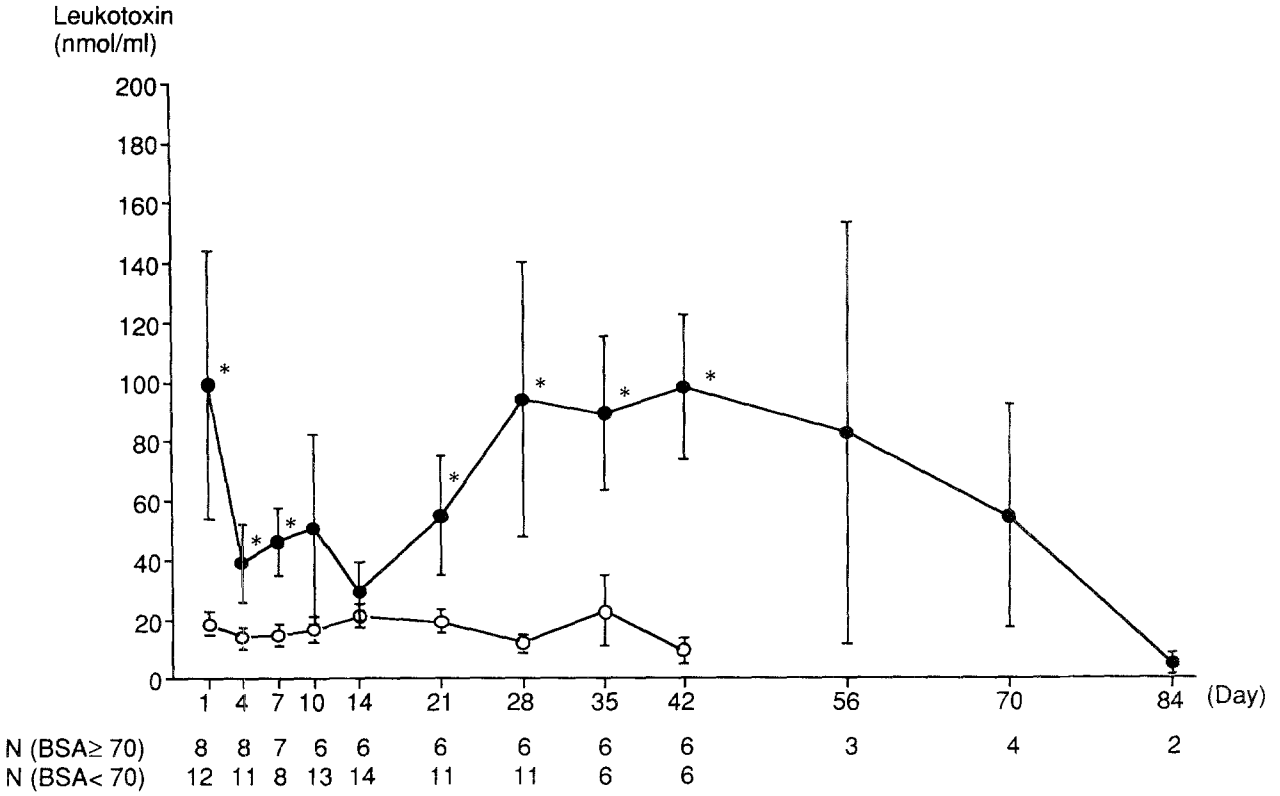

Fig. 1. Changes in plasma leukotoxin concentrations in patients with burn surface area over 70% (extensive burns) and in those with below 70%. Leukotoxin was detected throughout the investigation in patients with burns. In patients with extensive burns (solid circle), high leukotoxin concentrations were observed on the first day of injury, then leukotoxin decreased and it increased again 3 weeks after the injury. In patients with burn surface area below 70% (open circle), the increase in leukotoxin was significantly low (within a week and 3 weeks after the thermal injury) compared with the patients with extensive burns. As some patients were transported to our hospital several days after thermal injury and some patients were discharged within several weeks, the number of subjects analyzed differed period observed to period. BSA: burn surface area, *: $P < 0.05$.

Results

The mortality rate of patients with burn surface area over 70% was 66.7%, and it was significantly high ($P < 0.01$) compared with patients with burn surface area under 70% (the mortality rate; 22.2%). Fig. 1 shows the time course of plasma leukotoxin concentrations in patients with burn surface area over 70% (extensive burns) and in those with under 70%. In patients with extensive burns, on the first day of thermal injury the leukotoxin concentration was 99.6 ± 45.1 nmol/ml, the concentration then gradually decreased to 29.8 ± 9.6 two weeks after the injury. Thereafter, leukotoxin concentration increased to 98.7 ± 24.6, which was observed 6 weeks after the injury. Thereafter, the leukotoxin concentration decreased again. In patients with burn surface area under 70%, the increase in leukotoxin was significantly low (within a week and 3 weeks after the thermal injury) compared with the patients with extensive burns, and the maximum leukotoxin concentration, 22.6 ± 11.9, was observed 5 weeks after the injury. The mortality rate (61%) of patients with peak leukotoxin concentrations over 30 nmol/ml was significantly high ($P < 0.01$) compared with those below 30 nmol/ml (the mortality rate; 8%). In addition, the plasma leukotoxin concentration was below 2 nmol/ml in the control.

A significant correlation was found between plasma leukotoxin concentration in the early peak (the maximum value observed within 1 week) and burn surface area, log (leukotoxin nmol/ml) = 0.0143 × BSA (%) + 0.59, ($r = 0.57$, $P < 0.01$) as shown in Fig. 2a. Plasma leukotoxin concentration in the late peak (the maximum value observed after 1 week) was also correlated with burn surface area, log (leukotoxin nmol/ml) = 0.0145 × BSA (%) + 0.74, ($r = 0.72$, $P < 0.01$) (Fig. 2b). There was a significant correlation between plasma leukotoxin concentration in the early peak and that in the late peak, $y = 0.90\,x + 16.4$, ($r = 0.60$, $P < 0.01$) as shown in Fig. 3.

As shown in Fig. 4, the maximum concentration of leukotoxin in the late phase correlated significantly with that of C-reactive protein, log (leukotoxin nmol/ml) = 0.042 × C-reactive protein (mg/dl) + 0.74, ($r = 0.83$, $P < 0.01$), though there was no significant correlation between these two pa-

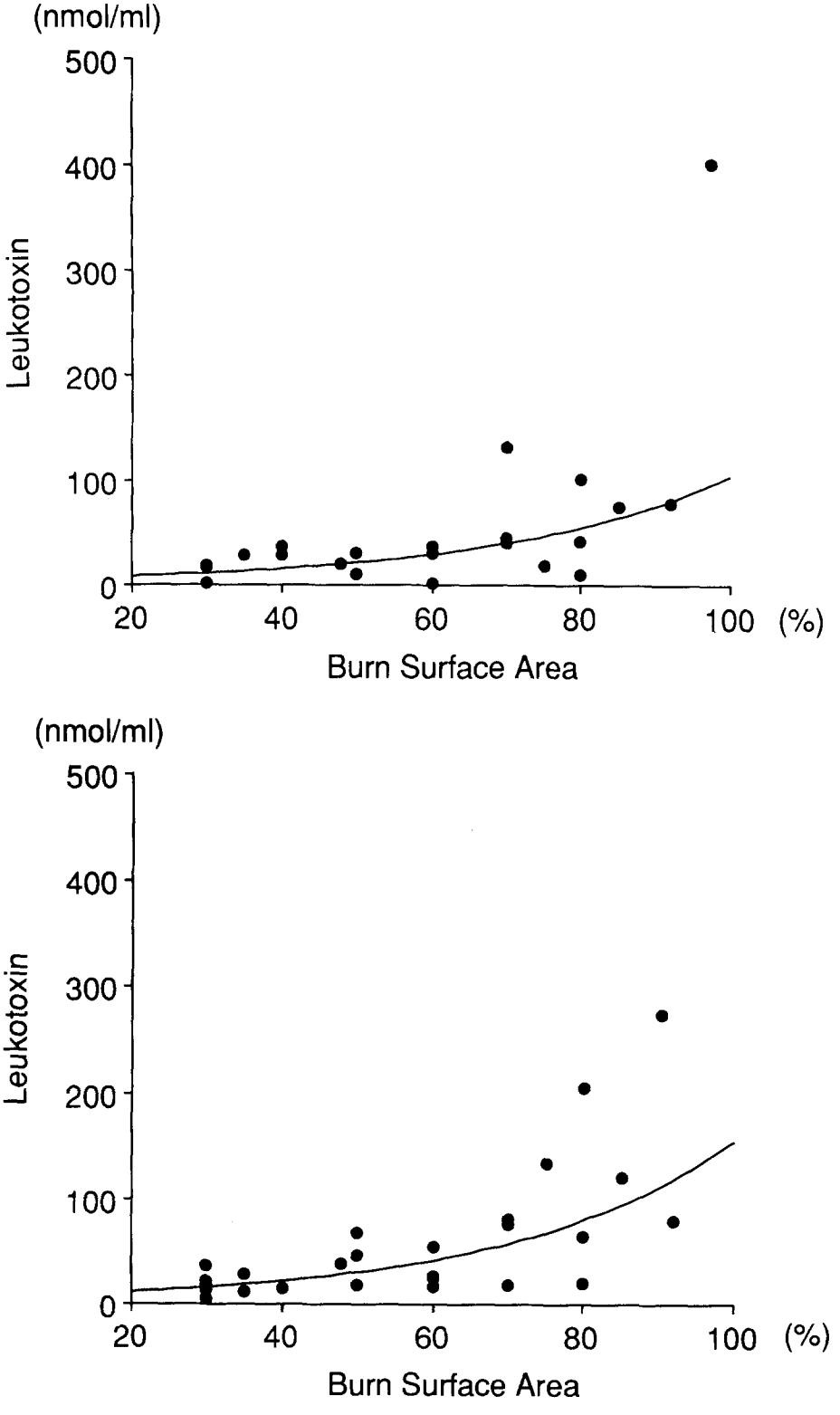

Fig. 2a and 2b. Correlations between the maximum plasma leukotoxin concentrations in the early phase and burn surface area (Fig. 2a, upper), and between those in the late phase and burn surface area (Fig. 2b, lower). Plasma leukotoxin concentrations significantly correlated with burn surface area, log (leukotoxin nmol/ml) = 0.0143 × BSA (%) + 0.59, ($r = 0.57$, $P < 0.01$) for the early phase and log (leukotoxin nmol/ml) = 0.0145 × BSA (%) + 0.74,($r = 0.72$, $P < 0.01$) for the late phase.

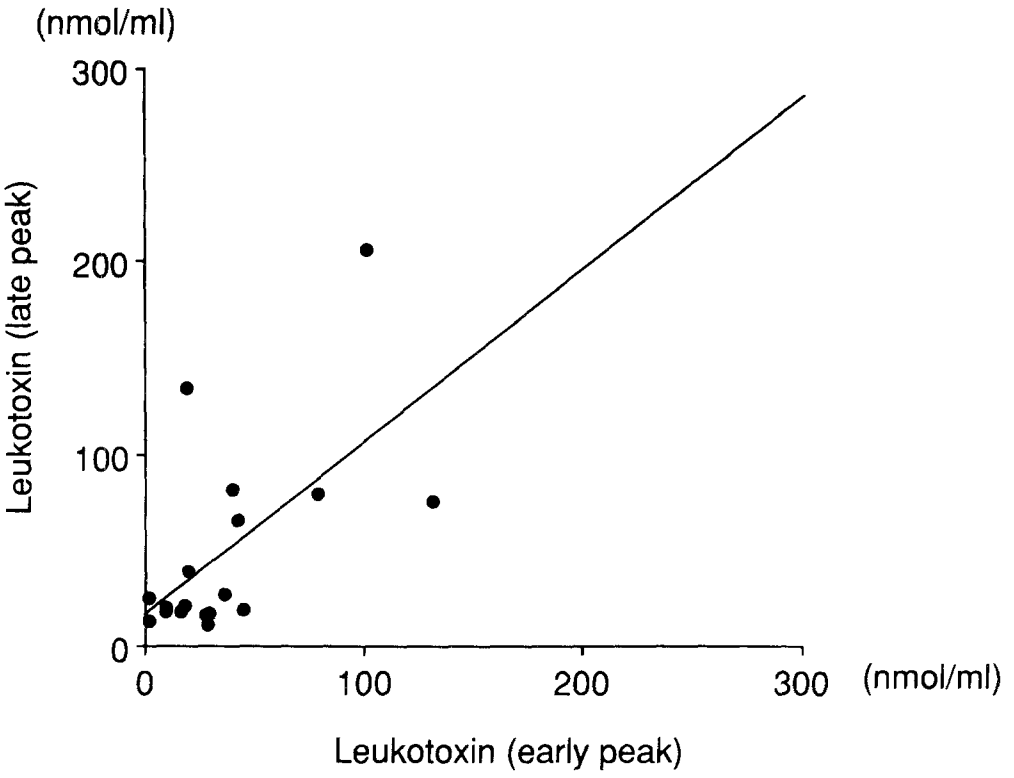

Fig. 3. Correlation between plasma leukotoxin concentrations in the early peak and those in the late peak. There was significant correlation between these two parameters, $y = 0.90\ x + 16.4$, ($r = 0.60$, $P < 0.01$).

rameters in the early phase.

The maximum plasma concentration of C-reactive protein in the late phase correlated significantly to burn surface area, $y = 0.30\ x + 2.50$, ($r = 0.75, P < 0.01$), though that in the early phase did not correlate significantly to burn surface area.

There is no significant correlation between the number of circulating white blood cells and leukotoxin concentrations in both the early (Fig. 5a) and the late phases (Fig. 5b). The number of circulating white blood cells did not correlate with burn surface area and C-reactive protein concentrations in both the early and the late phases.

Discussion

Leukotoxin has been revealed to decrease blood pressure, cardiac output, and left ventricular peak dP/dt in dogs, though administration of equivalent dose of linoleate, a precursor of leukotoxin, does not induce these hemodynamic changes [15]. In addition, death from cardiac failure was induced by administration of a large amount of leukotoxin in dogs [21]. It is also demonstrated that leukotoxin increases vascular permeability resulting in acute edematous lung damage [22], and that leukotoxin induces bleeding tendency [16]. These leukotoxin-induced pathological changes are characteristic features in patients with extensive burns. In the present study, an increase in leukotoxin was observed particularly in the late phase of thermal injury in patients with extensive burns, and plasma leukotoxin concentration correlated with burn surface area. In our *in vitro* study [12], we demonstrated that leukotoxin concentration over 30 nmol/ml exerted cellular damage. In the present study, a significantly high mortality rate was observed in patients with peak leukotoxin concentrations over 30 nmol/ml compared with those below 30 nmol/ml. Accordingly, the implication of leukotoxin is suggested in the pathogenesis of thermal injury. In addition, in the late phase, there was a good correlation between leukotoxin concentrations and C-reactive protein levels, which indicated that leukotoxin participated in the tissue destructive changes.

Neutrophils are major contributors to the self defense mechanism and the enzymatic reduction of molecular oxy-

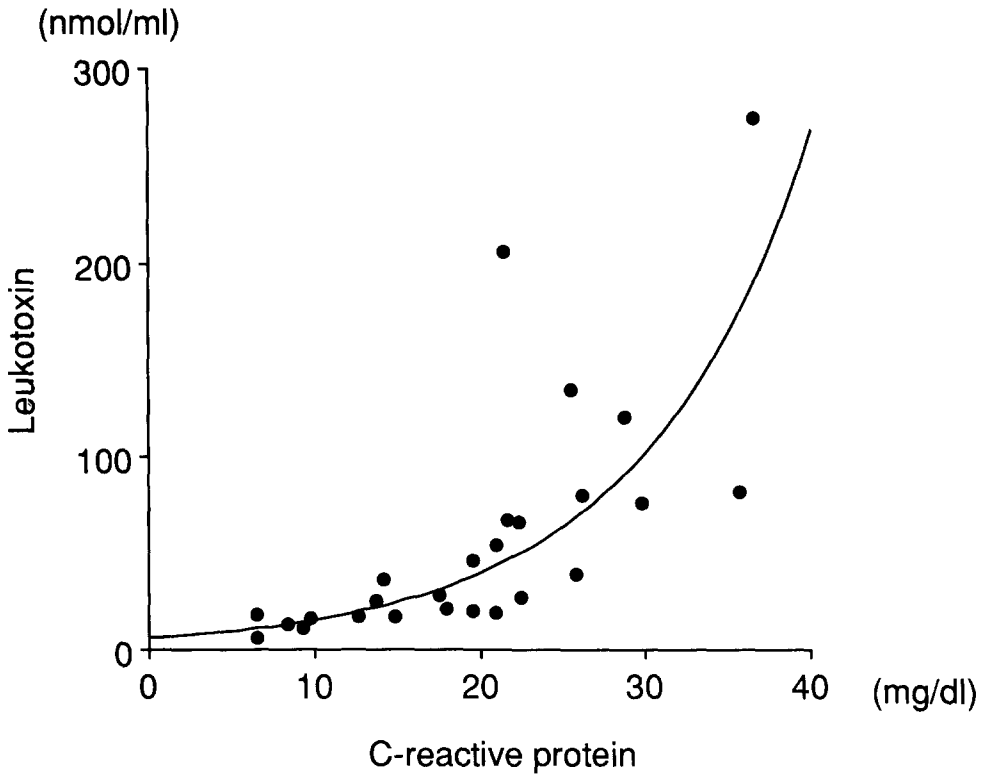

Fig. 4. Correlation between plasma leukotoxin concentrations in the late peak and those of C-reactive protein. Leukotoxin concentration correlated significantly to C-reactive protein concentration, log (leukotoxin nmol/ml) = 0.042 x C-reactive protein (mg/dl) + 0.74, ($r = 0.83$, $P < 0.01$).

gen, initially to superoxide anion and secondarily to other reduced forms such as hydroperoxide and hydroxyl radical, is major microbicidal mechanism in neutrophils. On the contrary, adverse effects of neutrophil-derived radicals are also recognized. Leukotoxin was produced from linoleate and hydroxyl radicals. Predominant existence of linoleate in the C-2 position of neutrophil membrane phospholipids (more than 60% in phosphatidylcholine and in phosphatidylethanolamine) has been reported by Yano and coworkers [23]. Under pathological conditions, phospholipase A_2 activity might be enhanced, resulting in an increase in the release of linoleic acid from cell membranes. Indeed, we have found a marked increase in polyunsaturated acid contents particularly linoleic acid content in burned skin [6]. Accordingly, there could be abnormal over-production of leukotoxin by neutrophils in such conditions. The same compound as leukotoxin was revealed to exist in rice plants as a self defense substance against fungal infection [24]. We also confirmed that leukotoxin has antifungal and antibacterial activities [7]. Accordingly, leukotoxin might be considered as an agent synthesized by neutrophils as part of the host defensive mechanism against various infectious diseases. However, over-production of leukotoxin by neutrophils under conditions of severe infection might cause tissue destruction. It has been believed that septic shock is a major contributory factor to the late death in patients with extensive burns. We pointed out here that neutrophils show not only protective effects against bacterial infection but also harmful effects to living cells, and neutrophil-derived leukotoxin might be involved in both mechanisms. In the present study, plasma leukotoxin concentration did not correlate with the number of circulating white blood cells, though an increase in the number of circulating white blood cells from normal upper limit was observed in 18 out of 22 patients in the early phase and in 16 out of 26 patients in the late phase. It is pointed out that without stimulation, neutrophils hardly synthesize leukotoxin, but with stimulation, neutrophils produce leukotoxin enormously [25]. That is, the number of circulating white blood cells itself might not correlate with the production of leukotoxin. Furthermore, the number of circulating white blood cells itself did not correlate with either C-reactive protein or with burn surface area.

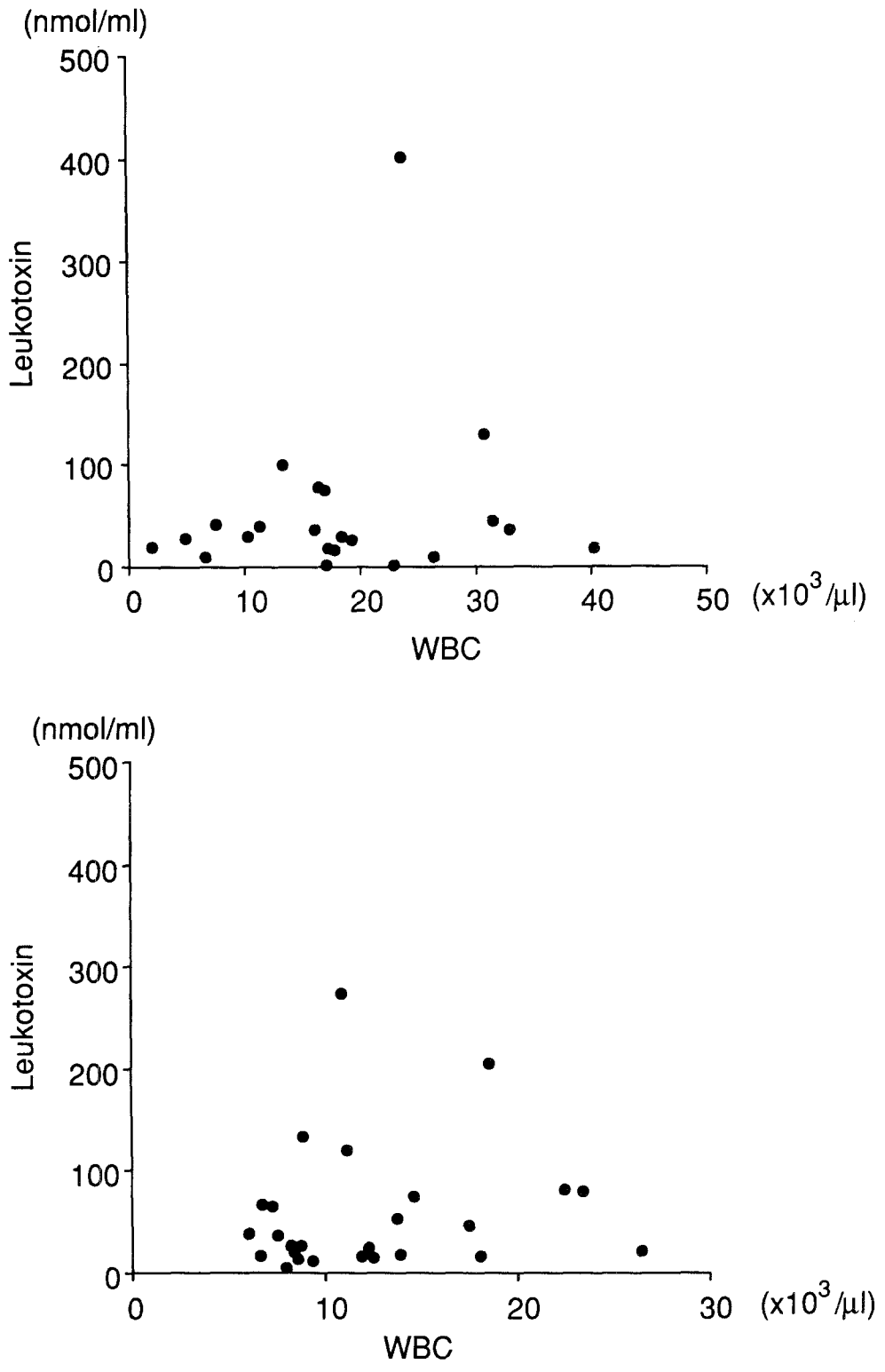

Fig. 5a and 5b. Correlations between the number of circulating white blood cells and leukotoxin concentrations in the early (Fig. 5a, upper) and in the late phases (Fig. 5b, lower). There were no significant correlations between these parameters in both phases.

The care of burned patients has progressed rapidly over the past 50 years. Before World War II, the average burn size associated with a 50% mortality rate in healthy young adults was less than 30% of the body surface. Today, the mean burn size associated with a 50% mortality rate in most burn centers ranges from 65–75% of the body surface [17]. In the present study, a 66.7% mortality rate was observed in patients with burn surface area over 70%, while patients with a burn surface area over 30% showed a 40% mortality rate. Although adequate infusion, increasing availability of antibiotics, and the advancement of surgical technique contribute to the improvement of eventual survival chance of patients with extensive burns, the development of multiple organ failure remains as the cause of death in patients with burns. Fruitful results might be obtained by the inhibition of leukotoxin synthesis.

In conclusion, leukotoxin concentration correlated well with burn surface area and leukotoxin might be a responsible and/or exacerbatory factor in various complaints associated with severe burns.

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