Functional Defects in Phagocytic Cells Following Thermal Injury

Application of Flow Cytometric Analysis

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Defective phagocytic cell function may partially account for the morbidity and mortality associated with thermal injury. In experimental thermal injury in the rat, small circulating blood volumes increase the difficulty in obtaining significant data. Furthermore, purification and or elicitation procedures have the potential for altering the cell surface characteristics and/or the functional response of the cell in question. We have examined the circulating neutrophils and pulmonary alveolar macrophages of anesthetized rats following a 16–20% body surface area scald injury to the shaved back. The circulating neutrophils of thermally injured rats were examined by flow cytometry following stimulation with phorbol myristate acetate (PMA) (100 ng/ml) in terms of the change in fluorescence intensity of the potentiometric cyanine dye, dipentyloxocarbocyanine and the formation of the oxidized product of 2',7'-dichlorofluorescin diacetate-loaded cells. The alveolar macrophages were examined after stimulation with PMA (100 ng/ml) in terms of the change in fluorescence intensity of the potentiometric dye, dipropylthiodicarbocyanine and the generation of superoxide production, as assessed by the superoxide dismutase inhibitable reduction of cytochrome *c*. Both cells exhibited a profound inhibition of cell function 4 hours after the insult, with partial return toward control values at later time points. Furthermore, the plasma of thermally injured rats, 4 hours after the burn was inhibitory to normal rat neutrophils. Fluorescent compounds suggestive of *in vivo* lipid peroxidation were maximally detectable at this time point. Further research is needed to establish the role of these products in the induction of phagocytic cell dysfunction. (Am J Pathol 1985, 118:116–127)

OVERWHELMING infection continues to be a major cause of morbidity and mortality following thermal injury despite considerable research in the field. Multiple defects in the immune response have been implicated potentially as contributing factors to the state of increased susceptibility to infection associated with thermal injury.[1] In addition, intrinsic[2–9] or extrinsic[10–13] defects in granulocyte and abnormal alveolar macrophage function[14] may be related to the abnormalities in host defenses of thermally injured patients.

Recent evidence has indicated that the bactericidal capacity of neutrophils and other phagocytic cells is significantly dependent on the integrity of the metabolic pathways responsible for the initiation of the "respiratory burst."[15] Although the sequence of biochemical reactions that precede the eventual formation of toxic oxygen radicals in phagocytic cells is not completely understood, several steps have been identified.[16] The temporal sequence of some of these reactions has been partially elucidated.[17] Thus, it is possible to measure the early changes indicative of cell stimulation by quantitating changes in the fluorescence intensity of dyes that measure the resting transmembrane potential and the relatively "late" responses of the activated phagocytic cell, namely, the generation of superoxide anion (O_2^-) or hydrogen peroxide.

In an attempt to determine whether experimental thermal injury is associated with abnormalities of the oxidative metabolism of rat circulating neutrophils and pulmonary alveolar macrophages, we have measured two related parameters: 1) the change in fluorescence

Supported in part by NIH Grants GM29507, GM28499, HL28442, and HL31963 and Coulter Electronics, Inc.

Accepted for publication July 31, 1984.

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intensity of two potentiometric cyanine dyes and 2) generation of oxygen radicals as assessed by two different techniques in response to stimulation by phorbol myristate acetate (PMA).[18,19] Our studies show the appearance of profound defects in blood neutrophils and alveolar macrophages from thermally injured rats. The data indicate that plasma from these animals will confer defects to neutrophils obtained from normal animals.

Materials and Methods

Materials

Ketamine sulfate was purchased from Parke-Davis (Morris Plains, NJ); 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was obtained from Eastman Kodak Co. (Rochester, NY); 3,3′-dipentyloxocarbocyanine (diO-C_5-[3]) and 3,3′-dipropylthiodicarbocyanine (diS-C_3-[5]) were obtained from Molecular Probes, Inc. (Junction City, Ore); PMA was from Consolidated Midland (Brewster, NY); cytochrome *c* (Type III) from horse heart, formyl-methionyl-leucyl-phenylalanine (FMLP) and superoxide dismutase were from Sigma Chemical Co. (St. Louis, Mo); and calcium ionophore A23187 was from Calbiochem-Behring (San Diego, Calif). All other materials employed were of reagent grade.

Buffers

The buffer used in the experiments designed to detect changes in fluorescence of cells equilibrated with diO-C_5-(3) or diS-C_3-(5) consisted of 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.8 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 22.5 mM Tris, 5 mM glucose, pH 7.4. The buffer used to detect the intracellular oxidation of DCFH consisted of phosphate-buffered saline (PBS) with 2 mM EDTA, 0.1% gelatin, and 5 mM glucose, pH 7.4.

Erythrocyte-Lysing Solution

The erythrocyte-lysing solution consisted of 0.15M NH_4Cl, 10 mM $NaHCO_3$, 10 mM EDTA, pH 7.4.

Experimental Animals

Adult, pathogen-free Long-Evans rats (250–350 g) from Charles River Laboratories were used throughout.

Thermal Injury

Following anesthesia (ketamine sulfate, 150 mg/kg intraperitoneally), the shaved backs of the rats were exposed to 90 C water for 30 seconds. This was accomplished with a Styrofoam template to which the rats were secured. The template had an area of approximately 100 sq cm (16–20% of total body surface area) that provided contact between the water and the shaved skin and shielded the rest of the body from contact with the thermal source. This allowed for a standard area of thermal injury in each case. The body surface area was calculated as follows[20]:

$$A = K \cdot W^{2/3}$$

where K = constant for the rat, W = weight in grams, and A = area in square centimeters.

Leukocyte Suspension

Leukocytes were obtained from peripheral blood, which was drawn from the inferior vena cava of anesthetized rats. Heparin was used at a final concentration in blood of 1 U/ml. The heparinized blood was diluted with a 30-fold excess of erythrocyte-lysing solution and constantly mixed at room temperature for 10 minutes. The cell suspension was then centrifuged at 400*g* for 5 minutes and washed twice with the appropriate buffer. The last centrifugation steps employed 200*g* for 10 minutes in order to remove platelets from the cell suspension. The final concentration of white blood cells was 5×10^6 cells/ml. Viability, as assessed by trypan blue exclusion, was >95%.

Alveolar Macrophages

Alveolar macrophages were harvested by bronchoalveolar lavage consisting of 15 washes with 8–10 ml warm (37 C) 0.9% NaCl solution. All of the animals were exsanguinated by sectioning of the major abdominal vessels following extraction of blood for obtaining plasma, and the lungs perfused through the right ventricle prior to bronchoalveolar lavage. Contaminating red blood cells in the lavage fluid were removed by exposure to a Tris-buffered ammonium chloride lysing solution. The cells were then washed with buffer and resuspended at the desired concentration. Viability, as assessed by trypan blue exclusion, was >95%.

Plasma Preparation

Where appropriate, plasma from normal and burned animals was obtained as follows: Blood was drawn from the inferior vena cava and mixed with heparin sulfate 1 U/ml (final concentration). The cells were removed by centrifugation (400*g* for 10 minutes). The supernatant plasma was withdrawn and centrifuged at 600*g* for 10 minutes for removal of platelets.

Measurement of the Change in Fluorescence Intensity of diO-C_5-(3)-Loaded Neutrophils

The loss of cell-associated fluorescence of diO-C_5-(3) has been demonstrated to be indicative of cell activation and presumably of a change in the resting transmembrane potential.[21] Additionally, cells of patients with chronic granulomatous disease are incapable of generating oxygen metabolites and show a decrease in the ability to undergo a change in the fluorescence intensity of diO-C_5-(3).[21] Thus, it would appear that there is a relationship between the change in fluorescence intensity of potentiometric dyes and the generation of oxygen metabolites.[17,22] In an attempt to define whether the loss of fluorescence of diO-C_5-(3)-loaded cells was abnormal following thermal injury, we performed the following experiments: the leukocytes (5×10^6 cells/ml) were suspended in the appropriate buffer and allowed to equilibrate with diO-C_5-(3) (2×10^{-9} M, final concentration) at 37 C for 5 minutes. The final concentration of ethanol in the cell suspension was 0.1%. The stimulus, PMA, 100 ng/ml (final concentration) or dimethylsulfoxide (DMSO), 0.1% (final concentration), was added, and the suspension was incubated at 37 C for 5 minutes. The cells were examined on an EPICS-C flow cytometer (Epics Division, Coulter Corporation, Hialeah, Fla) equipped with an argon laser at an excitation wavelength of 488 nm. Fluorescent emission (510–550 nm) was recorded with the photomultiplier (green) setting such that the normal unstimulated cell fluorescent histogram was at the upper limit of the 255 channels. The following parameters were recorded in each experiment in which 10,000 cells were examined; 1) forward light scatter (FLS), 2) log 90 degree light scatter (IL90), 3) linear green fluorescence (IGF), and 4) log green fluorescence (ILGF). An analog bit map was used to separate granulocytes from the lymphocytes and monocytes (Figure 1). The green fluorescence intensity was collected from the area delineated by the bit map. Although subtle light-scatter changes were detected in the stimulated cells, the granulocyte population was always within the confines of the bit map.

Measurement of the Intracellular Oxidation of 2′,7′-Dichlorofluorescin (DCFH) in Circulating Neutrophils

The cells were obtained as described above, resuspended in the appropriate buffer, and incubated at 37 C with DCFH-DA (5 μM, final concentration) for 15 minutes. The light scatter and green fluorescence parameters described above were used. The semi-quantitative evaluation of the amount of oxidized product 2′,7′-dichlorofluorescein (DCF) was as follows: the stimulated normal cells were examined after a 15-minute incubation at 37 C. The high voltage setting on the green photomultiplier tube was adjusted so as to visualize the highly fluorescent stimulated population beyond Channel 110 (of a total of 255 channels). At this same high voltage setting, duplicate or triplicate samples were analyzed, and 10,000 cells were examined in each experiment. Subsequently, cells in the absence of stimulus were similarly analyzed. Their profiles usually fell within the first 10 channels. Cells obtained from the burned animals were then examined with identical protocols. The results were expressed as the ratio of the percentage of the cell population beyond Channel 110 to the percentage of cells in Channels 10–110.

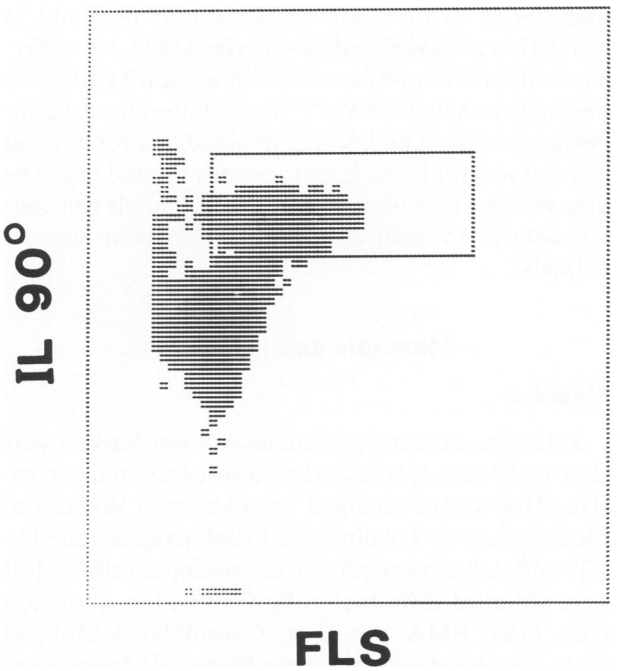

Figure 1 – Bit map gating of neutrophils. Ten thousand leukocytes were examined by light-scatter characteristics. FLS forward light scatter; IL90°, 90° light scatter (log scale). The granulocytes were gated by a bit map as shown.

$$\text{Channel ratio} = \frac{\%\text{ cells in channels } >110}{\%\text{ cells in channels } 10\text{–}110}$$

In the experiments designed to evaluate the effects of plasma on cell function, the same protocol was followed and the cells were resuspended in plasma after the 15-minute incubation with DCFH-DA in PBS. Upon addition of the stimulus, the cell suspension, in plasma, was examined under the conditions described above.

Detection of O_2^- in Alveolar Macrophages

Superoxide anion (O_2^-) was assayed by the superoxide dismutase inhibitable reduction of cytochrome *c* according to McCord and Fridovich following stim-

ulation with PMA.[19] Conversion to actual nanomoles of O_2^- was calculated with an extinction coefficient of 21.1 $cm^{-1}mM^{-1}$ for cytochrome *c* (reduced minus oxidized) at 550 nm. The results were expressed as the initial linear rate of O_2^- production (nanomoles per minute)/10^6 cells.

Detection of Changes in Transmembrane Potential of Alveolar Macrophages

Changes in transmembrane potential of alveolar macrophages were monitored with the potential sensitive cyanine dye (diS-C_3-[5]). Briefly, 1.5×10^6 alveolar macrophages were allowed to equilibrate with diS-C_3-(5), 2×10^{-6} M in a Varian S-330 spectrofluorometer at 37 C. Upon reaching a steady level of fluorescence intensity, PMA, 100 ng/ml (final concentration), was added. Following a brief lag period (30 seconds), an increase in fluorescence intensity was observed, and the difference (F_1-F_0) expressed as ΔF.

Detection of Fluorescent Compounds in Plasma

Normal, control, and thermally injured animals were anesthetized as described. Plasma was then obtained by the centrifugation of blood (obtained from the inferior vena cava) anticoagulated with 0.38% sodium citrate. Plasma from each experimental group (or time point after thermal injury) and control group was pooled and treated with 5% (final concentration) trichloroacetic acid for removal of proteins. The supernatant fluids were then filtered through membranes of 0.45-nm porosity, and 200 μl was examined by high-performance liquid chromatography on a reverse phase C_{18} column (MCH-10, Varian Instruments, Sunnyvale, Calif). The instrumentation consisted of a Varian LC 5020 chromatograph equipped with a Rheodyne 7/25 manual loop injector. The effluent was monitored at 210 nm (UV-50, Varian Instruments) and by fluorescence (Fluorichrom, Varian Instruments) with an excitation wavelength of 360 nm and an emission wavelength of 525 nm. The chromatogram was developed with a 20-minute gradient of 0–20% acetonitrile in H_2O containing 0.1% trifluoroacetic acid throughout, and followed by isocratic elution with 40% acetonitrile. The flow rate was 1.5 ml/min.

Results

Relation Between Intracellular Oxidized Product Expressed as Channel Ratio and Superoxide (O_2^-) Production in Neutrophils

The measurement of the formation of oxidized product by flow cytometry in circulating neutrophils of single animals is highly convenient, because this can be accomplished with small (<1 ml) samples of blood. However, due to wide fluorescence frequency distributions of the stimulated neutrophils previously loaded with DCFH-DA, we elected to express the results in a semiquantitative fashion as described in Materials and Methods. In order to establish the relationship of this semiquantitative method of expression of results to an established assay for oxygen radical production, we performed the following experiment: elicited neutrophils were obtained from the peritoneal cavity of several experimental animals 4 hours after the injection of glycogen (1%). The suspension was >95% neutrophils; and viability, as assessed by trypan blue exclusion, was >95%. The cells were pooled, and aliquots were studied either for O_2^- production (as assessed by the superoxide dismutase inhibitable reduction of ferricytochrome *c* or by flow-cytometric assay using the channel ratio method). Figure 2 illustrates a dose response using increasing concentrations of PMA and a comparison of the two methods. As the figure demonstrates, the two methods (which measure O_2^- and H_2O_2, respectively) resulted in dose-response profiles similar to that of PMA, with the flow-cytometric assay being somewhat more sensitive.

Analysis of Circulating Neutrophils From Normal and Thermally Injured Rats

Relative Granulocyte Counts

Following the thermal injury to rat skin, there is a marked neutrophilia with leukocytosis.[23] Compared with the controls, where circulating granulocytes comprise 15–25% of the total white count, thermally in-

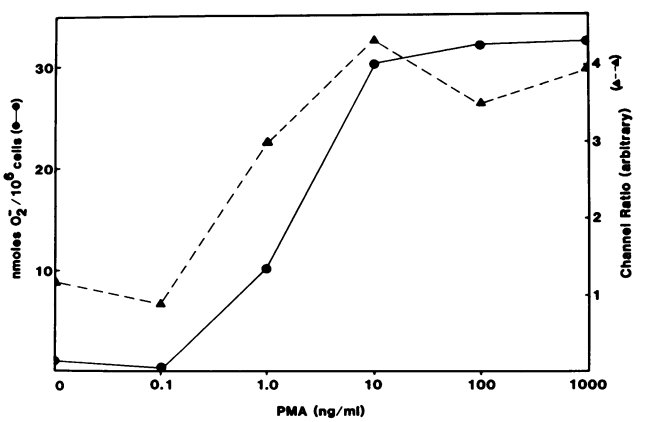

Figure 2—Comparison of channel ratio (by flow cytometry) to superoxide production (bulk cell assay) in peritoneal neutrophils; 1×10^6 neutrophils/ml were incubated in buffer with 80 μM cytochrome *c* in the presence (triplicates) and absence (triplicates) of SOD (23 μg/ml). Stimulation was with increasing concentrations of PMA (●—●). Channel ratio of neutrophils loaded with DCFH-DA, stimulated with increasing concentrations of PMA, and examined after a 15-minute incubation at 37 C (▲—▲).

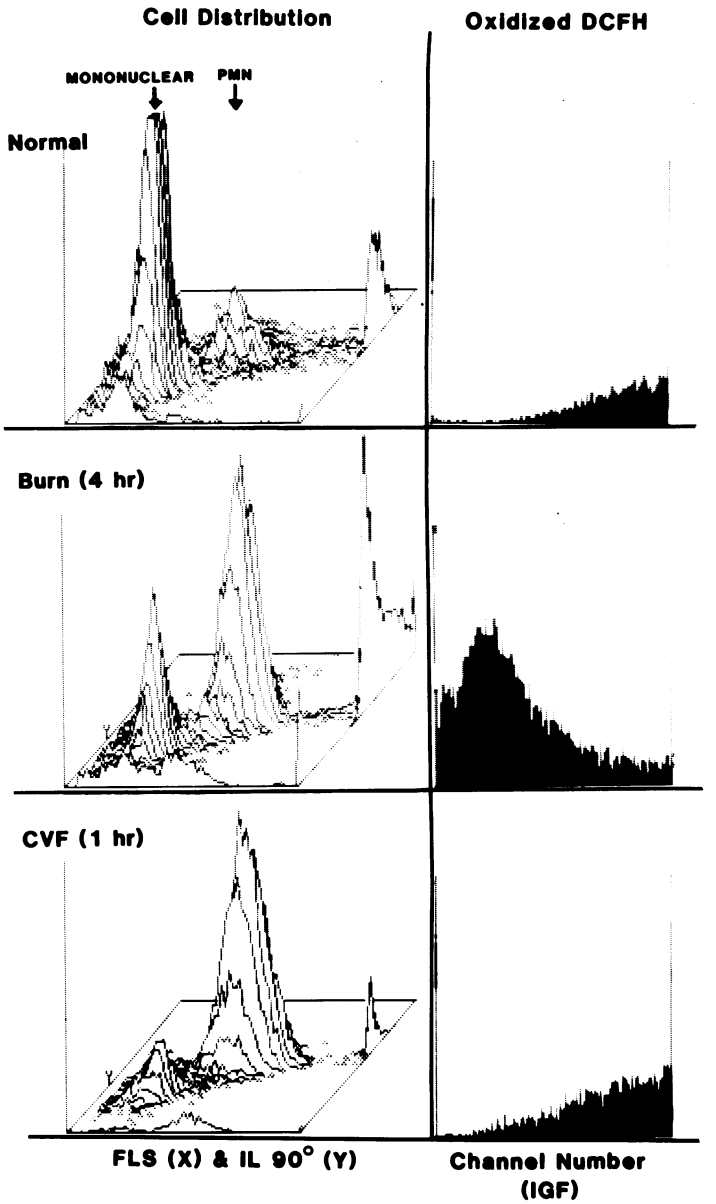

Figure 3—Effect of neutrophilia on the oxidation of intracellular DCFH. The frames on the left are isometric displays of FLS versus IL90 illustrating the changes in relative proportions of mononuclear and polymorphonuclear (*PMN*) cells in normal (*upper*), burn (4 hours) (*middle*) and following CVF (10 units) (*lower*). The frames on the right illustrate the fluorescence intensity of the bit-map-gated granulocytes previously loaded with DCFH-DA after stimulation with PMA (100 ng/ml) and incubation at 37 C for 15 minutes.

jured rats display an inversion of the relative proportions of granulocytes to lymphocytes 4 hours following the insult. It was important to determine whether changes in the functional capacity of granulocytes occurred under conditions of leukocytosis. To answer this question, we examined blood neutrophils from normal rats, from thermally injured rats (4 hours after injury), and from rats that had received 10 units of cobra venom factor (CVF) 1 hour previously. In the last group there is a marked neutrophilia following an initial neutropenia.[24] Figure 3 illustrates isometric distributions of peripheral leukocytes on the left and corresponding green fluorescence histograms of bit map gated granulocytes on the right. In the upper frame (left), the cell distribution of normal rat blood demonstrates approximately 20% of the cells as granulocytes. After stimulation with PMA as described in the Materials and Methods section, the green fluorescence histogram demonstrated the fluorescence to be in the high-numbered channels, indicative of intense fluorescence (Figure 3, upper frame, right). In contrast, in blood obtained from a thermally injured rat there was a marked granulocytosis (Figure 3, middle frame, left). When the granulocytes were examined following PMA stimulation, there was a considerable reduction in the amount of oxidized product, as demonstrated in a drop in the fluorescence intensity

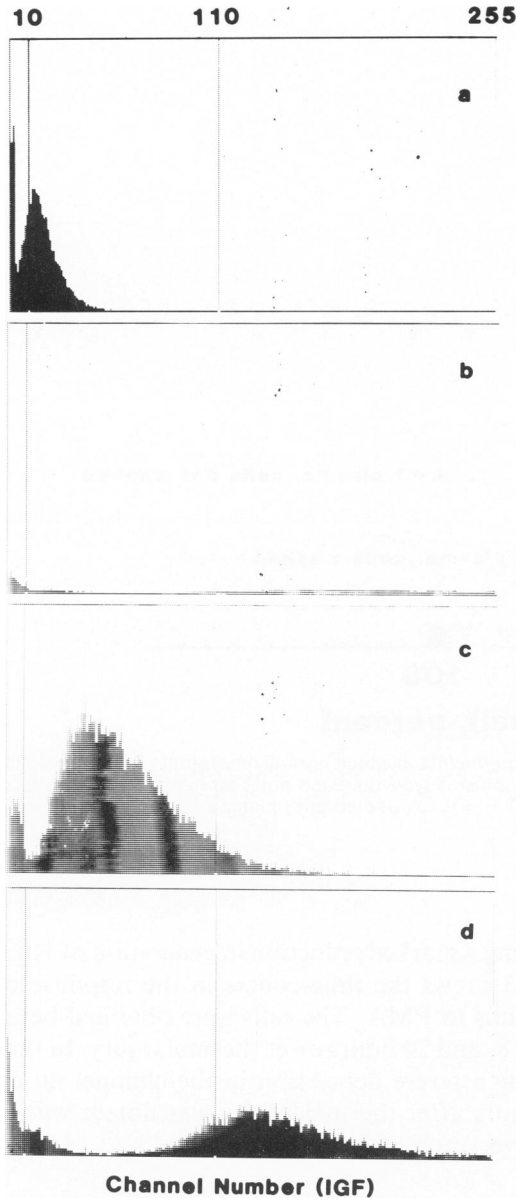

Figure 4—Typical fluorescence histograms obtained from 10,000 cells in each experiment; unstimulated (**a**); normal, stimulated with PMA (100 ng/ml) (**b**); stimulated neutrophils from thermally injured rats 4 hours after the insult (**c**); stimulated neutrophils from thermally injured rats 8 hours after the insult (**d**).

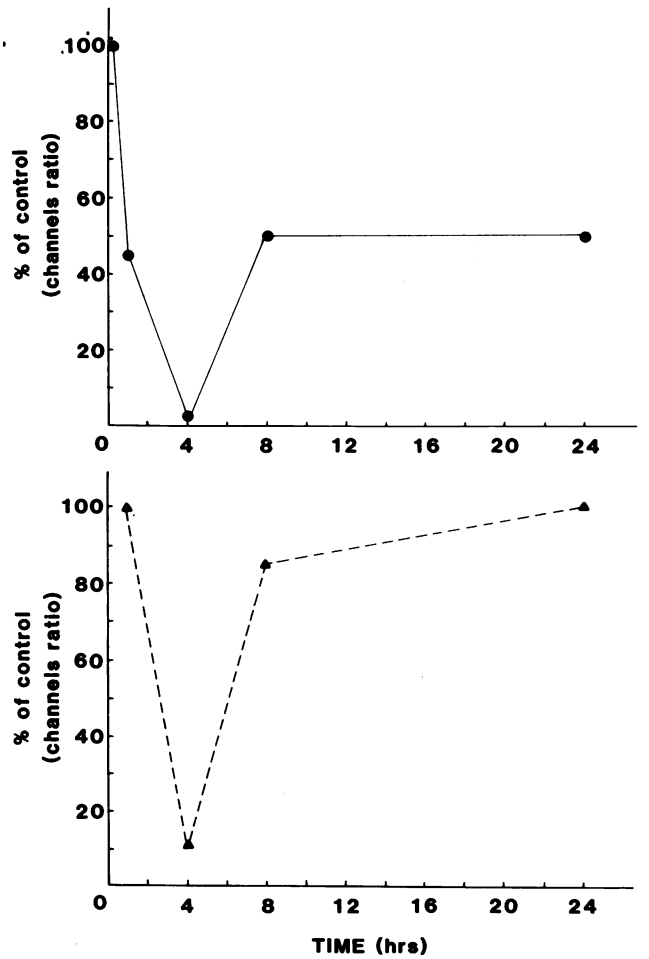

Figure 5—Time course of the effect of thermal injury on neutrophil function. *Upper frame*, channel ratio expressed as percentage of normal 1, 4, 8, and 24 hours after thermal injury. The neutrophils were obtained from thermally injured rats at the indicated time points. *Lower frame*, channel ratio expressed as percentage of control of normal neutrophils suspended in plasma obtained from thermally injured rats at the indicated time points. Each point is the mean of at least 10,000 determinations on individual cells in replicate experiments.

to the low-numbered channels (Figure 3, middle frame, right). To determine whether the reduced fluorescence was a consequence of the granulocytosis, we examined the leukocytes obtained 1 hour after infusion of CVF, which resulted in a marked granulocytosis (Figure 3, lower frame, left). However, when these cells were examined after stimulation by PMA, the fluorescence intensity (Figure 3, lower frame, right) was comparable to that of granulocytes obtained from the blood of normal rats (Figure 3, upper frame, right). Thus, the reduced formation of oxidized product in granulocytes of thermally injured rats would not appear to be a consequence of the granulocytosis.

It is possible that defective formation of DCF could be due to a decreased capacity of neutrophils to incorporate DCFH-DA and/or to hydrolyze DCFH-DA to DCFH. This was addressed as follows: blood was obtained from two normal rats and two thermally injured rats. The neutrophils were examined as described in the Materials and Methods section. Cells that had been obtained from the burned animals and preluded with DCFH-DA were incubated with exogenous H_2O_2 (50 μM). These cells, after addition of exogenous H_2O_2, displayed fluorescence intensity similar to cells har-

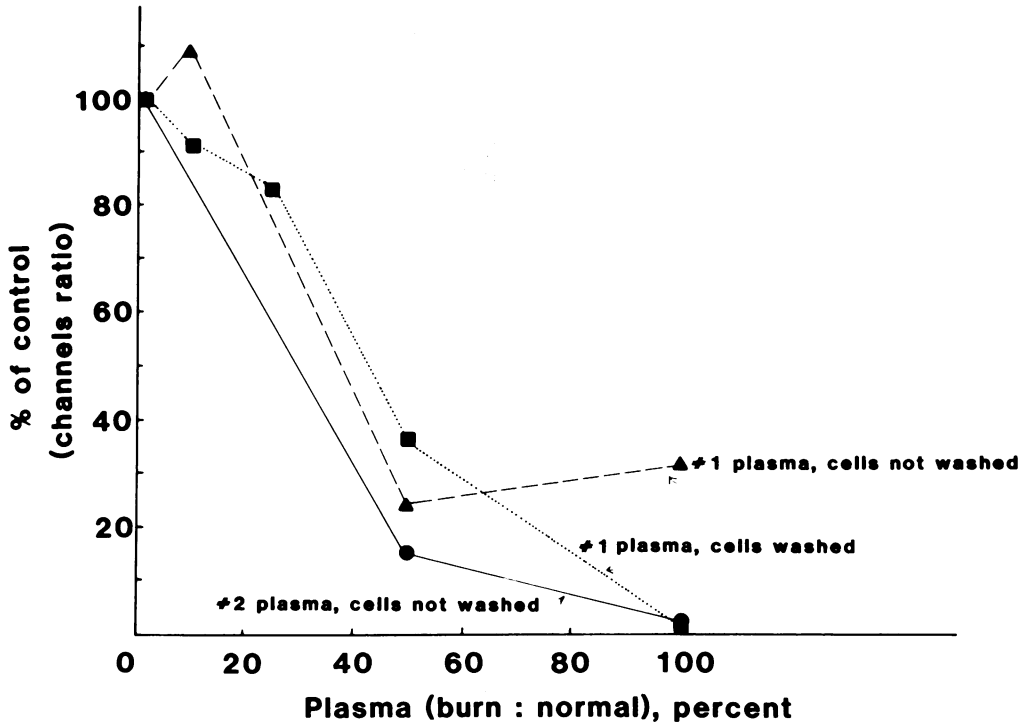

Figure 6 – Effect of burn plasma on normal rat neutrophils. Typical results of experiments in which normal neutrophils previously loaded with DCFH-DA in PBS were resuspended in varying proportions of normal and burn plasma (vol/vol). Each point represents the mean of at least 10,000 determinations of individual cells suspended in pooled burn plasma (O – – – O), pooled burn plasma from a separate experiment (▲– – –▲), pooled burn plasma, washed and resuspended in PBS (■- - - - -■).

vested from normal rats and similarly treated (data not shown). This suggests that the neutrophils from thermally injured rats can incorporate DCFH-DA as do cells from normal donors and subsequently hydrolyze DCFH-DA to DCFH. Thus, the defect in neutrophils of thermally injured rats is not due to an inability to load the cells with DCFH-DA or to defective hydrolytic activity.

Effect of Thermal Injury on PMA-Induced Oxidation of 2′,7′-Dichlorofluorescin by Neutrophils

We examined blood neutrophils of the animals 1, 4, 8 and 24 hours after the thermal injury. The green fluorescence of 10,000 neutrophils in each experiment was examined 15 minutes after stimulation with 100 ng/ml PMA. In each experiment the results were compared with those derived from cells obtained from normal rats. Figure 4 illustrates typical fluorescence histograms obtained from 1) normal, unstimulated neutrophils; 2) normal, stimulated neutrophils; 3) stimulated neutrophils obtained 4 hours after injury; and 4) stimulated neutrophils obtained 8 hours after injury. Cells obtained 4 and 8 hours after thermal injury are defective with respect to oxidation of DCFH-DA, suggesting a marked reduction in generation of H_2O_2. Figure 5 shows the time-course in the response of neutrophils to PMA. The cells were obtained before and 1, 4, 8, and 24 hours after thermal injury. In the upper frame a severe depression in the channel ratio 1 and 4 hours after thermal injury was noted, with partial recovery at 8 and 24 hours later.

Inhibitory Effects of Plasma From Thermally Injured Animals

To evaluate the effects of plasma from thermally injured animals on neutrophils obtained from normal rats, we suspended blood neutrophils (obtained from normal rats) in plasma taken either from normal or from thermally injured rats 1, 4, 8, and 24 hours after the injury. These cells had previously been incubated with DCFH-DA. As shown in Figure 5 (lower frame), the plasma from 4 hours after the thermal injury had marked inhibitory effects, indicating the ability of this plasma to render the cells defective with respect to H_2O_2 production.

The plasma obtained at the 4-hour interval was then diluted in varying proportions with normal plasma for determination of whether the inhibitory effect was dose-

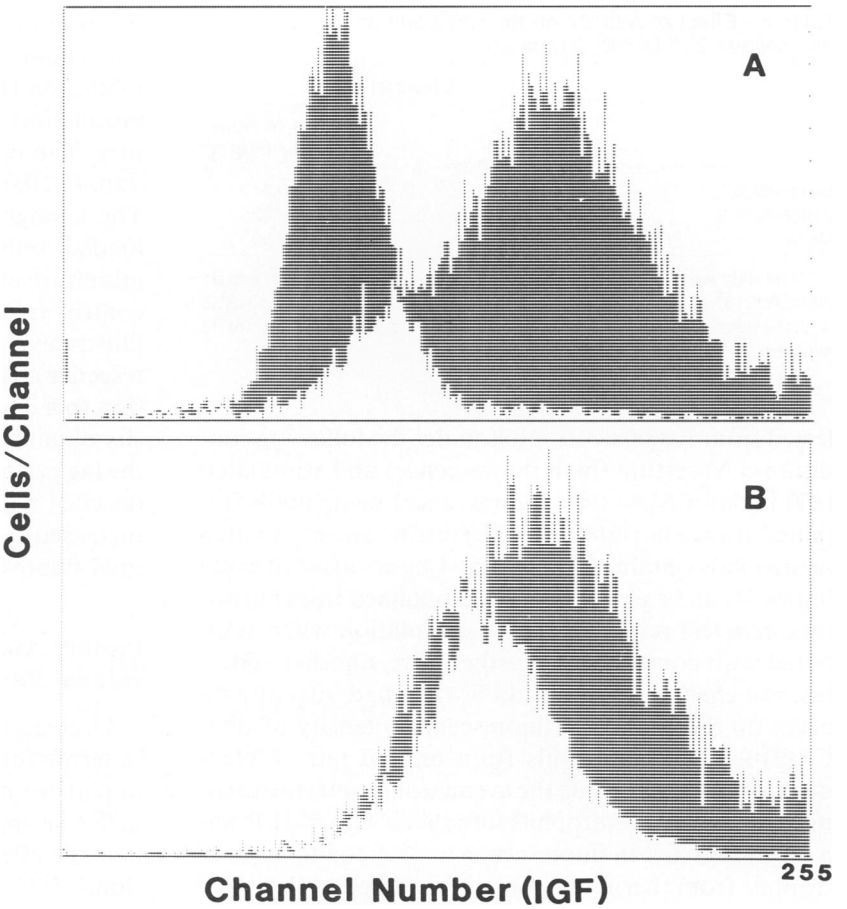

Figure 7—Effect of thermal injury on the fluorescence intensity of diO-C_5-(3) loaded neutrophils. **A**—Typical fluorescence frequency distribution of neutrophils obtained from normal rat, resting (*right*) and stimulated (*left*). **B**—Typical fluorescence frequency distribution of neutrophils obtained from thermally injured rats 4h after the insult; resting (*right*) and stimulated (*left*). Clear areas denote overlap. Depicted are the fluorescence (IGF) frequency distribution of 10,000 cells in each experiment.

related. Figure 6 illustrates the increasing inhibitory capacity of plasma from thermally injured rats. Figure 6 also demonstrates that the effect of burn plasma is not removed by washing of the cells with PBS. Because the cells were incubated with DCFH-DA for 15 minutes at 37 C in the presence of PBS and then resuspended in plasma, the decreased formation of DCF cannot be attributed to inhibitory effects of plasma on the uptake and hydrolysis of DCFH-DA.

Effect of Thermal Injury on the Change in Fluorescence Intensity of diO-C_5-(3)-Loaded Cells

The change in fluorescence intensity of neutrophils loaded with potentiometric cyanine dyes has been correlated with the onset of the respiratory burst.[17] Consequently, we determined whether thermal injury would be associated with a change in fluorescence intensity of neutrophils loaded with diO-C_5-(3) after stimulation with PMA. Figure 7 illustrates typical histograms col-

Table 1—Effect of FMLP on the Change in Fluorescence Intensity (ΔF) of Neutrophils Equilibrated With diO-C_5-(3) (2×10^{-9} M)*

	Control			Burn (4 hours after injury)		
	Resting	Stimulated	ΔF (of mean channel)	Resting	Stimulated	ΔF (of mean channel)
Experiment 1	162 ± 53.64	123 ± 58.2	−39	72.32 ± 32	79.55 ± 37.3	+7.23
Experiment 2	187 ± 77.09	155 ± 66.2	−32	54.37 ± 29.5	59.6 ± 30.0	+0.2

* Peripheral leukocytes were stimulated with FMLP (1×10^{-6} M) at room temperature. The neutrophils were examined after a 4-minute incubation at room temperature. Data are expressed as mean channel number ± SD.

Table 2—Effect of A23187 on the Oxidation of Intracellular 2',7'-Dichlorofluorescin

	Channel ratio*	
	Control	Burn (4 hours after injury)
Experiment 1	4.59	0.01
Experiment 2	2.93	0.02
Mean	3.62	0.02

* The data were obtained from 10,000 cells examined in each experiment. A23187 (2×10^{-5} M) was added to leukocytes suspended in HBSS + 0.1% gelatin. The cells were examined after incubation at 37 C for 15 minutes.

lected from 10,000 neutrophils under the following conditions: A) resting (high fluorescence) and stimulated (100 ng/ml PMA) (low fluorescence) neutrophils obtained from a normal rat and B) resting and stimulated neutrophils obtained from a rat 4 hours after thermal injury. It can be seen that the cells obtained from burned rats were less responsive after stimulation when compared with control cells. Furthermore, another stimulus, the chemotactic peptide FMLP, had virtually no effect on the change in fluorescence intensity of diO-C_5-(3)-loaded neutrophils from burned rats. Table 1 demonstrates that while the average of duplicate experiments in normal neutrophils stimulated with FMLP was a mean decrease in fluorescence of 35.5 channels, neutrophils from thermally injured rats failed to show evidence of membrane depolarization (fluorescence intensity increased by 3.7 channels). It is noteworthy that, in some experiments, the initial fluorescence intensity of burned neutrophils was lower than in controls. This could represent a difference in the actual resting transmembrane potential of neutrophils from thermally injured rats (data not shown).

Effect of Calcium Ionophore A23187 on the Intracellular Oxidation of DCF

The calcium ionophore A23187 is known to produce oxygen radicals in neutrophils in the presence of external Ca^{+2}.[25] We examined cells from normal and burned rats for the capacity of these cells to oxidize DCFH when stimulated by the calcium ionophore A23187 (2×10^{-5} M). Table 2 demonstrates a marked reduction in the formation of DCF. This suggests a relative nonspecificity of the cell defect following thermal injury.

Effect of Thermal Injury on Alveolar Macrophage Function

Table 3 illustrates the initial rate of superoxide (O_2^-) production and changes in fluorescence intensity (ΔF) upon stimulation with PMA (100 ng/ml) of alveolar macrophages retrieved by bronchoalveolar lavage 4, 6, and 8 hours following thermal injury. There was a 53% inhibition ($P < 0.001$) in the initial rate of superoxide production in cells obtained 4 hours after thermal injury. The inhibition was less at 6 hours (35% inhibition, $P < 0.005$) with a return to control levels by 8 hours. The changes in fluorescence intensity of diS-C_3-(5)-loaded cells followed a similar course, with 64% inhibition at 4 hours ($P < 0.005$), with a return toward control values at 6 and 8 hours after injury. Figure 8 illustrates a typical tracing from an experiment of fluorescence changes 4 hours after thermal injury. In addition to a decrease in the maximum fluorescence intensity obtained after stimulation with PMA (100 ng/ml), the lag period between addition of the stimulus and the onset of a rate of change in fluorescence intensity was increased, as was the time required to reach the maximal fluorescence intensity.

Further Analysis of Plasma From Thermally Injured Rats

Decreased opsonic activity has been demonstrated in serum following thermal injury.[12] This may account in part for neutrophil defects. However, in addition to defective opsonic activity, other investigators have found a toxic effect of burn serum on normal cell function.[11,26,27] The identity of this factor(s) has not been elucidated. We directed our attention to a report in recent literature by Nishigaki et al,[29] who reported on the presence of thiobarbituric acid-reactive material, suggestive of lipid peroxidation, in serum and multiple organs of animals subjected to thermal injury. Since the presence of fluorescent substances, a consequence of the formation of Schiff bases, is a measure of *in vivo* lipid peroxidation,[30] we assayed for the presence of these products in the acid-soluble fraction of plasma as described in Materials and Methods. Figure 9 illustrates

Table 3—Effect of PMA (100 ng/ml) on Superoxide (O_2^-) Production and Transmembrane Potential Changes (ΔF)*

		Time of cell harvest (after thermal injury)		
	Control	4 hours	6 hours	8 hours
O_2^-†	3.4 ± 0.2	1.6 ± 0.1 $P < 0.001$	2.2 ± 0.06 $P < 0.005$	3.4 ± 0.06 NS
ΔF‡	19.95 ± 2.7	7.13 ± 1.3 $P < 0.005$	25.0 ± 6.7 NS	16.7 ± 1.4 NS

* Values represent the mean ± SE of triplicate determinations of pooled alveolar macrophages obtained from 5 animals in each group.
† Nanomoles O_2^-/1 × 10^6 cells/min (initial linear rate of reduction of cytochrome *c*).
‡ Arbitrary units.

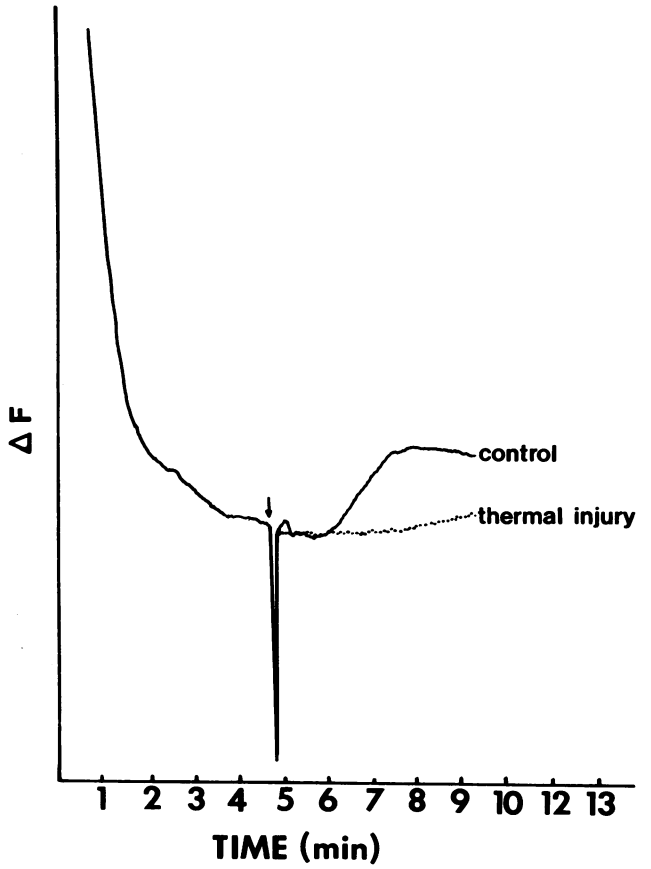

Figure 8 – Effect of thermal injury on the relative change in fluorescence intensity of diS-C_3-(5). Alveolar macrophages (1.5×10^6) were allowed to equilibrate with diS-C_3-(5), 2×10^{-6} M (final concentration). The *arrow* (↓) indicates the time of addition of PMA (100 ng/ml final concentration). The final concentration of DMSO was 0.1%.

the chromatograms obtained by reverse-phase HPLC from plasma derived at different time points after thermal injury. The fluorescent peaks were maximally present 4 hours after the burn. This time course is consistent with the appearance of thiobarbituric acid-reactive material described by Nishigaki et al,[29] and with the onset of the neutrophil defect. Further studies are needed to characterize the nature of these fluorescent products and their relation to cell dysfunction.

Discussion

The data presented above demonstrate a profound decrease in the capacity of circulating granulocytes and pulmonary alveolar macrophages from burned rats to respond to stimulation by PMA, as assessed by two different parameters (membrane depolarization and oxidative metabolism). Furthermore, a similar defect can be irreversibly produced in granulocytes obtained from normal animals, with the use of plasma obtained from thermally injured rats. Thus, the defect in neutrophils is at least partially mediated by a circulating substance(s). A contributing factor to the decrease in neutrophil function may be the transient decrease in circulating fibronectin.[12] However, in one report the transient depression in fibronectin could not be shown to have a direct effect on the evolution of the neutrophil dysfunction observed following thermal injury.[13] Our data would suggest a potential synergism between the presence of "toxic" factors and the decrease in nonspecific opsonic activity. The parallel time course of the appearance of fluorescent compounds, suggestive of lipid peroxidation, and the cell defect suggests but does not demonstrate a causal relation. These fluorescent compounds are probably a consequence of the reaction of malonaldehyde with amino acids or their esters to yield conjugated Schiff bases (N-C=C-C=N) which have typical electronic absorption and fluorescence properties.[30] Together with measurement of pentane, the measurement of fluorescent products constitutes a sensitive method for the detection of *in vivo* lipid peroxidation.[31] These fluorescent products have also been detected in conditions associated with altered inflammatory mechanisms such as preeclampsia[32] and in the synovial fluid of arthritic patients.[33] Further investigation is needed, however, for the establishment of the role that these fluorescent compounds play in producing cellular dysfunction.

Other investigators have also found in neutrophils from thermally injured subjects defects related to the respiratory burst cycle. Specifically, Heck et al have shown decreased levels of NADH-NADPH oxidase activity[8] and decreased O_2 consumption[4] in neutrophils

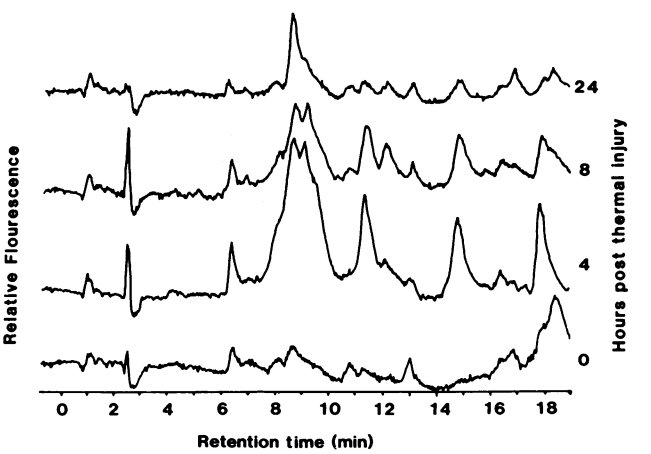

Figure 9 – Effect of thermal injury on the appearance of fluorescent compounds in plasma. The TCA-soluble fraction plasma was prepared as described in Materials and Methods. The efferent was detected with the use of a fluorimeter with an excitation wavelength of 360 nm and an emission wavelength of 525 nm.

from burned humans. The stimulated nitroblue tetrazolium reduction test, which relies on the formation of a formazan derivative, indicates defective oxygen radical production in neutrophils from patients with chronic granulomatous disease (CGD).[34] Similar data have also been reported in neutrophils of thermally injured patients.[35] Chemiluminescence, partially indicative of singlet oxygen formation, was shown by Schmidt et al to be defective in neutrophils of burned rats.[36] Thus, a number of reports would substantiate the results reported in this article. On the basis of the data presented above, it is not possible to exclude the possibility that changes measured in circulating neutrophils reflect a state of cell "exhaustion" following maximal stimulation. Alternatively, there is the potential for direct thermal injury to leukocytes circulating through the portion of skin that comes in contact with the thermal source. Although we have made no attempt to quantitate or estimate the percentage of neutrophils potentially stimulated and/or injured directly in this fashion, this possibility cannot be disregarded. The fact that plasma from burned animals can transfer the defect to cells obtained from normal rats would suggest that the cells are rendered defective because of the presence of inhibitory substances present in the plasma.

The reduction in the initial rate of reduction of cytochrome *c* and thus of superoxide anion (O_2^-) generation by alveolar macrophages following stimulation with PMA is consistent with previously published data in a similar burn model involving rats.[14] Loose and Turinsky showed a depression in oxygen consumption following stimulation of alveolar macrophages with PMA at similar time points (4 hours) after burn. The reduction in the change in fluorescence intensity and in its rate in diS-C_3-(5)-loaded alveolar macrophages is suggestive of a defect involving the activation of the respiratory burst. Defects such as those seen in patients with CGD[17] or pharmacologically induced defects of phagocytic cells[22] are also associated with a decreased response to PMA as assessed by both parameters described above. It is reasonable, then, to assume that similar mechanisms could be operative in alveolar macrophages. Further research should clarify the nature of the phagocytic defects following thermal injury and the role of putative circulating depressants of phagocytic cell function.

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Acknowledgments

The authors would like to thank Mr. Wallace Tourtellotte for his excellent technical assistance and Ms. Jeny Breakstone for typing the manuscript.