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Biochemical and Functional Alterations in Macrophages After Thermal Injury

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Biochemical and functional measurements of rat pulmonary alveolar macrophages were measured 4 h after 1 10-s, 26 to 28% total body surface area, full-thickness scald burn induced under ether anesthesia. Both phagocytic activity and capacity were significantly decreased to a comparable extent, whereas microbicidal activity was increased almost twofold in macrophages from the burned animals. Concurrent with the decreased phagocytic function was a marked impairment in chemotaxis and random migration of these cells when zymosan-activated serum was used as the chemoattractant. When biochemical parameters were examined, it was demonstrated that, on a per-cell but not total-protein basis, alveolar macrophages from burned animals had elevated levels of RNA, total protein β-glucuronidase, acid phosphatase, and 5′-nucleotidase. These results raise the possibility that the increased pneumonitis in burned individuals may be due to more complex macrophage dysfunctions than impaired microbicidal activity, as was once thought. Alternatively, the biochemical and functional changes observed may be a reflection of a new population of macrophages appearing in the lungs after thermal injury.

Approximately two-thirds to three-fourths of the deaths which occur after burn injury have been attributable to infections (31, 32). The incidence and severity of these infections are significantly influenced by the age of the patient and the size of the burn.

The increased susceptibility to infection, beyond that which might be expected to occur simply from the loss of the skin barrier, has been related to an impairment in host defense mechanisms (25) and can be modified by synthetic immunomodulators (34). This increased susceptibility can be demonstrated within a few minutes after burning, when the burn wound is still sterile (1). If germfree mice, which have a low mortality after thermal injury, are "normalized" by the introduction of *Escherichia coli* into their gastrointestinal tracts, their mortality after burn injury approaches that of conventional mice (21), demonstrating the importance of endogenous flora in the postburn septic episode.

Perhaps the most common cause of postburn death is pneumonitis, which has been attributed to impaired host resistance (32). Although it was initially speculated that the pneumonitis was due to an impaired pulmonary alveolar macrophage microbicidal activity, alveolar macrophages were demonstrated to have a normal microbicidal activity 4 h postburn and, indeed, an enhanced microbicidal activity 24 h after burn injury (18). However, during this same time, their phagocytic activity was profoundly decreased; the impaired phagocytic activity was, in part, serum mediated. Coincident with the decreased phagocytic function was a decreased respiratory burst (19). These composite studies suggested that perhaps the altered pulmonary host resistance after thermal injury is not due to an impaired macrophage microbicidal function but instead to a phagocytic defect. Another possibility is that a chemotactic deficiency exists which may be expressed either as an in situ defect or a systemic mobilization defect or both. Dominioni et al. (8) recently demonstrated that the in vivo chemotactic response of polymorphonuclear leukocytes labeled with indium-111 after isolation from burned guinea pigs was comparable to that observed with polymorphonuclear leukocytes from normal animals. In addition, it was observed that the polymorphonuclear leukocytes from the burned donor left the circulation more rapidly and localized in the burn area and the liver. No evidence of increased destruction of polymorphonuclear leukocytes from burned donors by the spleen or lungs was observed. However, the relationship between this observation and postburn pneumonitis is still unclear. Since alveolar macrophage chemotactic activity has not been previously examined after thermal injury, the present studies were undertaken to examine the postburn in vitro chemotactic activity of pulmonary alveolar macrophages and to ascertain whether it correlated with certain biochemical and functional parameters of the macrophages and could be considered an additional causal factor in postburn pneumonitis. The underlying hypothesis was that an activation of pulmonary alveolar macrophages occurs after thermal injury, but that the generation of a putative soluble mediator inhibited membrane-related events, e.g., phagocytosis and chemotaxis.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (190 to 210 g) were used throughout. Purina lab chow and water were provided ad libitum, and a 12:12 day-night photoperiod was maintained. All animals were fasted overnight before the study.

Burn injury. A full-thickness scald burn on 26 to 28% of the total body surface area was produced by immersing the dorsum of ether-anesthetized rats in a 90°C water bath for 10 s. A control group was anesthetized in ether but not burned.

Cell isolation. Alveolar macrophages were obtained by pulmonary lavage with isotonic saline. Three 10-ml amounts of isotonic saline were infused into the lungs of ether-anesthetized rats through a tracheal cannula. The lungs were massaged in situ for 2 min, and the pulmonary lavage fluid was then withdrawn through the cannula. This procedure was conducted three times for each animal, and all three

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pulmonary aspirates were pooled and kept on ice. The pooled lavage fluid was centrifuged at 200 × *g* for 5 min at 4°C in siliconized conical centrifuge tubes. The cell pellet was suspended in Hanks balanced salt solution (HBSS). A differential cell count with Wright stain and uptake of neutral red revealed that the suspension contained ca. 90% macrophages and 5% small lymphocytes with a 95% viability as determined by trypan blue dye exclusion.

Phagocytic and microbicidal studies. The technique used to assess the phagocytic and microbicidal activity of murine phagocytic cells is an adaptation (33) of a light-microscopic procedure initially described by Schmid and Brune (30), who used human peripheral blood neutrophils. Briefly, a phagocyte-yeast (*Saccharomyces cerevisiae*) ratio of 1:3 was utilized. Sterile capped plastic test tubes (12 by 75 mm) which held 0.20 ml of a solution containing 25.6 mg of methylene blue per 100 ml of HBSS, 0.25 ml of the cell suspension in HBSS, 0.25 ml of yeast in serum, and 0.10 ml of HBSS were incubated at 37°C in a Dubnoff shaking water bath for 30 min. Preliminary studies conducted to adapt the method of Schmid and Brune, which used human cells, to a murine system revealed optimal activity at 30 min. This time was then chosen as the time for measurement. The tubes were centrifuged at 3.2 × *g* for 6 min at 4°C after removal from the water bath. The supernatant was discarded, and the cell pellet was suspended in 20 μl of HBSS. A wet mount was prepared for light-microscopic examination, and a minimum of 200 phagocytic cells were counted per preparation. Dead yeast cells stained blue with the methylene blue, whereas live yeast cells remained unstained. The percentage of macrophages which contained at least two yeast cells was used as an index of phagocytic activity. The total number of yeast cells, live and dead, ingested per 100 phagocytic cells was tabulated and expressed as phagocytic capacity. The total number of dead yeast cells, i.e., those which stained blue, within the macrophages was counted and expressed as a function of cellular microbicidal activity. All phagocytic and microbicidal studies were conducted in duplicate. Phagocytic activity, capacity, and killing were determined by the following equations:

$$\text{Phagocytic capacity} = \frac{\text{number of live and dead yeast cells}}{\text{100 cells}}$$

$$\%\ \text{killed} = \frac{\text{number of dead yeast cells}}{\text{phagocytic capacity}}$$

$$\text{Phagocytic activity} = \frac{\text{number of cells containing 2 or more yeast cells}}{\text{100 cells}}$$

Biochemical measurements. The determination of total cell protein was based upon the method of Lowry et al. (20) as modified by Miller (22), with bovine serum albumin as the standard protein. Beta-glucuronidase was measured with a Sigma kit (Sigma Chemical Co., St. Louis, Mo.; Tech. Bull. no. 325 [1-75]) based on the procedure described by Fishman et al. (12), modified by using an incubation temperature of 56 instead of 37°C. Acid phosphatase was measured by the method described by Lentz and DiLuzio (16) without any modification. 5'-Nucleotidase activity was determined with a Sigma kit (Tech. Bull. no. 675). RNA was measured by the procedure of Burton (6) as modified by Richards (29), in which the pellet is solubilized in perchloric acid and read directly at 260 nm.

Chemotaxis. The chemotaxis procedure was employed as initially described by Synderman and Stahl (35), with zymosan-activated serum as the chemotactic factor. Chemotaxis chambers (no. 200-187) were obtained from Neuro Probe Inc. (Bethesda, Md.). Permeable polycarbonate filters 10-μm thick with 5-μm pores were purchased from Nuclepore Corp. (Pleasanton, Calif.) and used as top filters. Methylcellulose filters with 0.45-μm pores were obtained from Millipore Corp. (Bedford, Mass.) and served as the cell-impermeable bottom filters.

The chemotactic factor used in all assays was zymosan-activated normal male CBA/J mouse serum standardized for maximal response. Complement-derived chemotactic factors were produced by activation of serum by zymosan. Stock zymosan suspension (1 mg/ml) was prepared from zymosan (lot 2455; Nutritional Biochemicals Corp., Cleveland, Ohio) which had been standardized for complement activation of normal human serum assessed by evaluating both chemotactic factor production and conversion of C3 by crossed immunoelectrophoresis. The stock zymosan suspension was centrifuged in a Serofuge for 5 min, and the pellet was suspended in mouse serum at a concentration of 1.3 mg of zymosan per ml of serum and then incubated for 60 min at 37°C with mixing every 15 min. The zymosan was then pelleted in a Serofuge, and the serum was removed by aspiration and heated at 56°C for 30 min to inactivate unconverted complement components and noncomplement-derived, heat-labile chemotactic factors. Each serum pool was then divided into aliquots and stored at −70°C until used.

The zymosan-activated serum was added to the lower chamber at a volume of 0.2 ml. The isolated alveolar macrophages were added to the upper chamber at a concentration of 1.5×10^6/ml, with a volume of 0.2 ml being used. The chambers were incubated for 4 to 5 h at 37°C in a humidified container. After incubation, the chambers were removed, and the lower filters were fixed in ethanol, stained with Harris hematoxylin and erosin stain, cleared in xylene, and mounted with Permount on slides under cover slips as described by Boyden (5). The filters were examined under high power (430×), and the number of mononuclear cells per high-power field was determined. The filter was scanned horizontally and vertically through the center of the filter, and the number of cells in a grid in each of 10 high-power fields was recorded. The average number of cells per grid was multiplied by a correction factor of 1.8 (the ratio of the number of cells per high-power field to the number of cells per grid) to obtain the average number of cells per high-power field.

Statistical evaluation. Data are presented as the mean ± standard error of the mean. Significance was determined by the Student *t* test.

RESULTS

Alveolar macrophages obtained by pulmonary lavage 4 h after thermal injury had a twofold increase in total cellular protein. The total protein of macrophages from burned animals was 399.41 ± 73.09 $\mu g/10^6$ cells, whereas control animals had 154.44 ± 28.88 $\mu g/10^6$ cells (Table 1). Associated with this marked increase in total protein was a concomi-

TABLE 1. Biochemical characterization of pulmonary alveolar macrophages after thermal injury[a]

Group	Total protein[b] (μg/10^6 cells)	β-Glucuronidase (Sigma units/10^6 cells)	Acid phosphatase (μM phosphate/10^6 cells)	5′-Nucleotidase (μg of phosphate/10^6 cells)	RNA (μg/10^6 cells)
Control (n = 14)	154.44 ± 28.88	45.56 ± 8.95	0.06 ± 0.01	6.99 ± 1.17	3.71 ± 0.43
Burn (n = 6)	399.41 ± 73.09[b]	119.60 ± 12.79[b]	0.14 ± 0.01[b]	15.38 ± 1.85[b]	5.50 ± 0.24[b]

[a] Macrophages were harvested by pulmonary lavage 4 h after a 10-s, 90°C, 26 to 28% body surface area scald burn. All data are presented as the mean ± standard error.
[b] $P < 0.05$.

tant elevation in RNA, in that macrophages from burned animals had 5.50 ± 0.24 μg of RNA per 10^6 cells, and controls had 3.7 ± 0.43 μg of RNA per 10^6 cells. Also associated with the increased RNA and protein in macrophages from burned animals was an elevation in the two lysosomal enzymes, beta-glucuronidase and acid phosphatase. The beta-glucuronidase activity of macrophages from burned animals was 119.60 ± 12.79 Sigma units per 10^6 cells, whereas in controls it was only 45 ± 8 Sigma units per 10^6 cells. Acid phosphatase was similarly increased to 0.14 ± 0.01 μM phosphate per 10^6 cells in macrophages from burned animals, in contrast to a control value of 0.06 ± 0.01 μM phosphate per 10^6 cells. The ectoenzyme membrane marker 5′-nucleotidase was also increased twofold on a per-cell basis in the burn macrophages when compared with control values (Table 1). However, as can be observed in Table 1, the total protein (micrograms per 10^6 cells) was increased more than twofold in macrophages from burned animals. Therefore, when the enzyme values and RNA content were calculated on a protein basis, there were no significant differences between control macrophages and macrophages from thermally injured rats.

Consistent with the increased per-cell biochemical parameters observed in macrophages obtained from burned animals was an approximate twofold increase in their microbicidal activity (Table 2). However, in contrast to the enhanced microbicidal activity, alveolar macrophages from burned animals had significantly reduced phagocytic activity as well as reduced phagocytic capacity. This reduction was essentially half of that seen with macrophages from control animals, and the decreased phagocytic activity observed with alveolar macrophages was comparable to that previously demonstrated with peritoneal macrophages (19).

The most dramatic defect observed was the reduction in chemotactic activity of alveolar macrophages obtained from burned animals. This marked impairment in chemotaxis was noted at each time interval examined, i.e., at 4, 24, and 48 h. Random migration was similarly impaired; however, by 48 h there was no statistically significant difference in random migration of controls versus cells obtained from burned animals (Table 3).

The cell yield, differential, and viability of the pulmonary alveolar macrophages obtained from thermally injured rats were always comparable to those observed when control animals were used.

DISCUSSION

The increased incidence and severity of infection in burned individuals has been attributed to an impairment in their immunologically mediated host defense systems (2). This immune suppression may be related to an altered cell-mediated immunity (23–25, 38), soluble serum immunosuppressive factors (15), opsonin defects (13), endocrine alterations (36, 37), or neutrophil (3, 7) and mononuclear cell dysfunctions (18, 19). Perhaps one of the more important consequences of the impaired host defense status in burned individuals is the pneumonitis which has been reported to be the most common cause of postburn death (32).

Since the pulmonary alveolar macrophage is an integral component of pulmonary host defense, previous studies examined its fucntion after thermal injury. It was demonstrated that alveolar macrophage microbicidal activity was enhanced, but phagocytic activity was significantly decreased (18). Since rat alveolar macrophages phagocytize bacteria only through the Fc receptor, as opposed to human, rabbit, and hamster alveolar macrophages, in which phagocytosis is Fc and C3 receptor mediated (26), it may be speculated that the phagocytic defect in alveolar macrophages from burned rats may in part be related to a defect in Fc binding or activation or both. Furthermore, the respiratory burst, but not resting O_2 consumption, was also found to be markedly impaired (19), in agreement with the altered phagocytic state. Similarly, in the present study, with a 10-s burn as opposed to the 30-s burn previously used, enhanced microbicidal but decreased phagocytic function was also demonstrated (Table 2). However, further examination of alveolar macrophage function also revealed profound impairment in chemotactic activity when zymosan-activated serum was used as the chemoattractant (Table 3).

Numerous studies of impaired leukocyte chemotaxis have been described after thermal injury (11, 14). Indeed, it has been reported that the depression of in vitro chemotaxis correlates with the burn surface area and even with patient survival (4, 14). Furthermore, this impairment in chemotaxis may precede the septic episode (4) and is a transient defect in patients who survive (4, 11). In contrast to the previously cited chemotactic studies, which used either neutrophils (11, 14) or peripheral blood monocytes (4), the present study utilized alveolar macrophages, which also demonstrated a profound chemotactic defect (Table 3). This defect lasted at

TABLE 2. Phagocytic and microbicidal activity of pulmonary alveolar macrophages after thermal injury[a]

Group	Phagocytic capacity[b]	Phagocytic activity	Microbicidal activity
Control (n = 14)	183.3 ± 11.7	61.7 ± 4.4	9.6 ± 3.6
Burn (n = 10)	95.0 ± 21.1[b]	40.7 ± 7.3[b]	15.9 ± 3.3[b]

[a] Pulmonary alveolar macrophages were harvested by pulmonary lavage 4 h after a 10-s, 90°C, 26 to 28% body surface area scald burn. All data are presented as the mean ± standard error.
[b] $P < 0.05$.

TABLE 3. Pulmonary alveolar macrophage chemotactic defect after thermal injury[a]

Group	Chemotaxis at hour:			Random migration at hour:		
	4	24	48	4	24	48
Control	25.7 ± 3.8	28.8 ± 5.8	21.7 ± 6.6	10 ± 1.3	13.1 ± 2.7	16.3 ± 3.4
Burn	5.3 ± 0.09[b]	2.9 ± 0.81[b]	3.7 ± 0.11[b]	1.7 ± 0.02[b]	2.5 ± 0.10[b]	12.8 ± 4.2

[a] Chemotaxis and random migration were measured at 4, 24, and 48 h. The chemotactic stimulus used was zymosan-activated serum. All data are presented as cells per high-power field ($\bar{X}$ ± standard error). $n = 9$ in all studies.
[b] $P < 0.05$.

least until 48 h postburn (the time of the last measurement) and did not appear to be improving. Random migration was also defective; however, by 48 h it had returned to control values.

Concomitant with defective chemotaxis was a significant increase in all the biochemical parameters measured, i.e., RNA, protein, beta-glucuronidase, acid phosphatase, and 5'-nucleotidase, in the macrophages from thermally injured animals when expressed on a per-cell basis. Since lysosomal enzyme activity was not decreased, one could speculate that the chemotactic defect observed in the present study was perhaps not due to an in vivo deactivation. Previous studies have demonstrated the disappearance of 5'-nucleotidase from the surface of activated macrophages (9, 10). Another possibility is that rat alveolar macrophages require an accessory cell to promote chemotactic responsiveness, as has been demonstrated in patients with mycosis fungoides (28) and with mouse macrophages (17). If this accessory cell, even in small numbers, is depleted, then its absence may account in part for the impaired responsiveness. In addition, the possiblity of the presence of serum inhibitors (15) must be included, based on the inhibitory effect of burn serum reported by Ninneman et al. (27).

Further studies are being conducted to explore the specificity of the chemotactic defect as well as the influence of putative serum inhibitory components and accessory cell requirements.

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