INFECTION AND IMMUNITY, June 1972, p. 938-941

Vol. 5, No. 6
Printed in U.S.A.

# Selective Effects of Thermal Injury on Mouse Peritoneal Macrophages

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Received for publication 4 February 1972

Normal mice or mice burned 6 days previously were injected intraperitoneally with either saline or 50 μg of concanavalin A. Four days after the injection the peritoneal macrophages were collected and examined in vitro. Macrophages from concanavalin A-injected mice appeared activated because several in vitro parameters of activity were increased. Examination of macrophages from burned animals revealed a selective depression of macrophage activity.

The functional state of macrophages following thermal injury is essentially unknown. Reticuloendothelial (RES) function is apparently depressed because decreased vascular clearance of colloidal particles (20, 21, 24) and abnormal distribution of injected bacteria (10) have been observed under experimental conditions in burned animals. However, alterations in macrophage activity could not be clearly demonstrated in these studies because other factors, such as vascular changes after thermal injury, conceivably could influence the results of tests of RES function.

The present study was undertaken in an effort to evaluate macrophage activity after thermal injury. Five features of peritoneal macrophages were evaluated in vitro–phagocytosis, the degree of color change of neutral red-dyed *Candida albicans* after phagocytosis, spreading on glass, adherence to glass, and activation following intraperitoneal injection of concanavalin A (Con A).

## MATERIALS AND METHODS

Female, 18- to 20-g CFW mice were burned by dipping the ether-anesthetized animals into a water bath at 96 C for 3 sec. Approximately 30% of the surface area was immersed. The resulting scald produced a third-degree burn with 5% mortality by 10 days. Control animals were anesthetized but not burned.

The phagocytic ability of macrophages from control and burned animals was tested in vitro. Macrophages were collected by washing the peritoneal cavity with 3 ml of Hanks balanced salt solution (HBSS). The concentration of macrophages was determined in a hemocytometer after supravital staining with neutral red. A suspension (0.5 ml) of $2.5 \times 10^5$ macrophages was placed on an 18-mm round cover glass and incubated at 37 C for 15 min. The unattached cells were removed by rinsing the cover glass in HBSS. Phagocytosis was evaluated in the following ways. (i) A suspension of $10^6$ neutral red-dyed heat-killed *Candida albicans* (NR *Candida*) (12) in 0.5 ml of medium 199 containing 20% heat-inactivated (56 C for 30 min) fetal calf serum was placed on the cover glass. The cultures were incubated at 37 C for 30 min in an atmosphere of 5% $CO_2$ in air with intermittent swirling agitation. The monolayer was washed free of extracellular NR *Candida* and mounted in HBSS on a microscope slide. The number of *Candida* per 200 macrophages and percentage of the intracellular NR *Candida* that had turned red were determined. The ability of the macrophages to change the amber-colored NR *Candida* to a red color after phagocytosis (expressed as the percentage of red intracellular NR *Candida* at 30 min) varies with different functional states of the cell (C.W. Smith and A.S. Goldman, Exp. Cell Res., *in press*). This color change has been interpreted as an indication of an acidic *p*H in the phagosome (12). (ii) Rabbit red blood cells (RBC) were prepared from freshly collected blood by washing them three times in HBSS. A suspension of $10^6$ washed rabbit RBC in 0.5 ml of medium 199 containing no serum was placed over the monolayer of macrophages. The cultures were incubated at 37 C for 30 min in an atmosphere of 5% $CO_2$ in air. Extracellular RBC were removed by rinsing with HBSS, and the macrophages were fixed for 30 min in 1% glutaraldehyde in 0.1 M phosphate buffer (*p*H 7.2). The cover slips were mounted in buffer and examined with a Reichert interference-contrast microscope. The number of RBC in 200 randomly selected macrophages was determined. Rabbit RBC are phagocytized much more actively by mouse peritoneal macrophages following stimulation in vivo with phytohemagglutinin, serum (7), or Con A (C.W. Smith and A.S. Goldman, Exp. Cell Res., *in press*), and phagocytosis does not seem to be dependent on a serum opsonin. (iii) Mouse (CFW) RBC were prepared from freshly collected blood by washing three times in HBSS. A suspension

of $10^6$ washed mouse RBC in medium 199 containing a 1:64 dilution of rabbit anti-mouse (CFW) RBC serum was placed over the macrophages. The cultures otherwise were handled exactly as when rabbit RBC were used as phagocytic particles.

Spreading of macrophages on glass was evaluated by a modification of North's technique (17). Macrophages were collected and suspended in HBSS as described above. A suspension of $5 \times 10^4$ cells in 0.5 ml of HBSS was placed over an 18-mm cover slip and incubated at room temperature for 10 min. The unattached cells were removed by rinsing in HBSS, and the cover slips were immersed in medium 199 preheated to 37 C. Incubation was continued at 37 C in an atmosphere of 5% $CO_2$ in air for 15, 30, or 60 min. The cells then were fixed in glutaraldehyde solution for 30 min and mounted in buffer. By using an eyepiece micrometer and Nomorski optics (100× objective), two diameters (the longest cell diameter and the longest diameter perpendicular to it) were determined for each of 200 macrophages per culture. The average of these two measurements was used as an estimate of the cell diameter. Since maximum spreading occurred after 30 min of incubation at 37 C in both burned and control animals, only the data collected at this time interval will be given.

Activation of macrophages was accomplished by intraperitoneal (ip) injection of 50 μg of Con A, prepared by the method of Sumner and Howell (23), suspended in 0.5 ml of 0.85% saline solution. This has been shown to result in the appearance of macrophages which have, at 4 days postinjection, increased capability of producing red intracellular NR *Candida*, increased phagocytic ability, and increased spreading on glass (C.W. Smith and A.S. Goldman, Exp. Cell Res., *in press*). All control animals were injected ip with 0.5 ml of 0.85% saline solution, and the macrophages were collected 4 days later.

## RESULTS

The burned area consisted of a dry, brown eschar with no gross evidence of infection by 10 days after the scald, and the burned mice weighed an average of 2.5 g less than the unburned controls of the same age. Deaths from thermal injury usually occurred within the first 2 days postburn.

The total number of cells recovered from the peritoneal cavity of burned mice was slightly higher ($P < 0.1$) than controls 5 days postburn. No difference in cell recovery was noted at 7 or 10 days, though. Burned and normal mice injected with Con A yielded a significantly higher cell recovery 4 days after the injection. Examples of these data can be given for mice in the following treatment groups. From mice injected 6 days postburn with Con A, a mean of 4,200 cells/$mm^3$ was recovered, whereas, from mice injected 6 days postburn with saline, a mean of 2,700 cells/$mm^3$ was recovered ($P < 0.01$). From normal mice injected with Con A or saline, the mean cell recovery was 4,800 and 3,000 cells/$mm^3$, respectively ($P < 0.05$). The peritoneal cells in each group were collected 4 days after injection of Con A or saline. Differential counts were not significantly different.

The capability of peritoneal macrophages to change NR *Candida* from an amber to a red color after phagocytosis was determined in the four treatment groups given above as well as in mice injected with Con A or saline 1 or 3 days postburn (macrophages were collected 4 days after the injection as in the above groups). In macrophages from mice burned 1 and 3 days

TABLE 1. *Percentage of neutral red-dyed heat-killed Candida that turned red after phagocytosis by peritoneal macrophages from normal mice and mice with thermal injury to the skin*

| Exptl groups | Stimulant[a] | Scald (days)[b] | Mice (no.) | Mean % ± SD[c] |
|---|---|---|---|---|
| 1 | Saline | None | 7 | 45.5 ± 8.1 |
| 2 | Saline | 1 | 8 | 42.2 ± 12.0 |
| 3 | Saline | 3 | 4 | 51.4 ± 9.2 |
| 4 | Saline | 6 | 6 | 26.5 ± 11.2 |
| 5 | Con A | None | 6 | 74.4 ± 10.2 |
| 6 | Con A | 1 | 9 | 69.4 ± 10.6 |
| 7 | Con A | 3 | 6 | 64.0 ± 14.2 |
| 8 | Con A | 6 | 6 | 54.1 ± 9.5 |

[a] Macrophages collected 4 days after ip injection of the stimulant. Con A = concanavalin A.

[b] Time of scald before ip injection of the stimulant.

[c] Statistical comparisons (Student's *t* test): groups 1 and 5, $P < 0.001$; 1 and 4, $P < 0.001$; 5 and 8, $P < 0.005$; 4 and 8, $P < 0.001$. SD = standard deviation.

TABLE 2. *In vitro phagocytosis of neutral red-dyed heat-killed Candida by peritoneal macrophages from normal mice and mice subjected to thermal injury of the skin*

| Exptl groups | Stimulant[a] | Scald (days)[b] | Mice (no.) | *Candida* per 200 macrophages ± SD (mean no.)[c] |
|---|---|---|---|---|
| 1 | Saline | None | 7 | 78.1 ± 11.6 |
| 2 | Saline | 6 | 6 | 74.1 ± 13.4 |
| 3 | Con A | None | 8 | 110.0 ± 10.5 |
| 4 | Con A | 6 | 6 | 116.2 ± 16.5 |

[a] Macrophages collected 4 days after ip injection of the stimulant. Con A = concanavalin A.

[b] Time of scald before ip injection of the stimulant.

[c] Statistical comparisons (Student's *t* test), groups 1 and 2, *P* not significant (NS); 3 and 4, *P* NS: 1 and 3; $P < 0.001$; 2 and 4, $P < 0.001$. SD = standard deviation.

TABLE 3. *In vitro phagocytosis of rabbit and mouse red blood cells (RBC) by peritoneal macrophages from normal mice and mice subjected to thermal injury of the skin*

| Exptl groups | Stimulant[a] | Scald (days)[b] | RBC | Mice (no.) | RBC per 100 macrophages ± SD (mean no.)[c] |
|---|---|---|---|---|---|
| 1 | Saline | None | Rabbit[d] | 9 | 18.1 ± 14.0 |
| 2 | Saline | 6 | Rabbit[d] | 6 | 12.5 ± 14.2 |
| 3 | Con A | None | Rabbit[d] | 8 | 160.2 ± 54.9 |
| 4 | Con A | 6 | Rabbit[d] | 9 | 119.2 ± 75.4 |
| 5 | Con A | None | Mouse | 6 | 1 |
| 6 | Con A | None | Mouse[e] | 6 | 92.6 ± 30.1 |
| 7 | Con A | 6 | Mouse | 6 | 2 |
| 8 | Con A | 6 | Mouse[e] | 6 | 100.1 ± 28.4 |

[a] Macrophages collected 4 days after ip injection of stimulant. Con A = concanavalin A.
[b] Time of scald before ip injection of stimulant.
[c] Statistical comparisons (Student's *t* test): groups 1 and 2, *P* not significant (NS); 3 and 4, *P* NS; 1 and 3, $P < 0.001$; 2 and 4, $P < 0.001$; 6 and 8, *P* NS. SD = standard deviation.
[d] Culture medium contained no serum.
[e] Cultures contained a 1:64 dilution of antimouse RBC serum prepared in rabbits.

TABLE 4. *Spreading on glass of peritoneal macrophages from normal mice and mice subjected to thermal injury of the skin*

| Exptl groups | Stimulant[a] | Scald (days)[b] | No. of Mice | Mean diameter ± SD[c] |
|---|---|---|---|---|
| 1 | Saline | None | 5 | 13.0 ± 1.6 |
| 2 | Saline | 6 | 5 | 12.8 ± 2.1 |
| 3 | Con A | None | 5 | 21.0 ± 1.8 |
| 4 | Con A | 6 | 5 | 16.2 ± 1.7 |

[a] Macrophages collected 4 days after ip injection of the stimulant. Con A = concanavalin A.
[b] Time of scald before ip injection of the stimulant.
[c] Statistical comparisons (Student's *t* test): 1 and 2, *P* not significant; 3 and 4, $P < 0.005$. SD = standard deviation.

before injection of Con A or saline, the percentage of red, intracellular NR *Candida* was not significantly different from unburned controls (Table 1). However, in macrophages from mice burned 6 days before injection of saline or Con A, the percentage of red, intracellular NR *Candida* was significantly reduced for both saline and Con A-treated groups when compared with unburned controls (Table 1).

In contrast to the reduced capacity for changing the NR *Candida* to a red color after phagocytosis, the level of phagocytosis of *Candida* was not influenced by the burn (Table 2). As seen in Table 3, the degree of phagocytosis of RBC also was not significantly altered in burned animals.

In addition to the evaluation of phagocytosis, the number of macrophages in five randomly selected low-power microscope fields was determined for each of the cultures. Con A-stimulated macrophages remained on the cover glass in significantly higher numbers than saline-stimulated macrophages ($P < 0.001$). The burn injury was associated with a significant decrease in the number of glass-adherent macrophages only in Con A-injected mice (402 ± 50 for burned, Con A-injected mice versus 523 ± 63 for normal, Con A-injected mice, $P < 0.005$ and 300 ± 38 for burned, saline-injected mice versus 277 ± 45 for normal, saline-injected mice, *P* not significant).

Table 4 presents the results of macrophage spreading experiments. Con A resulted in increased spreading, and the degree of this increased spreading was significantly lower in burned, Con A-injected mice.

## DISCUSSION

The results in this and a previous report (C.W. Smith and A.S. Goldman, Exp. Cell Res., *in press*) are consistent with the interpretation that Con A causes an increase in the activity of peritoneal macrophages in normal mice. This is evidenced 4 days after ip injection of 50 μg of Con A by an increased number of macrophages recovered from the peritoneal cavity, an increased phagocytosis of *Candida*, rabbit RBC, and mouse RBC, an increased percentage of red NR *Candida* 30 min after phagocytosis, an increased area of spreading on glass, and an increased number of glass-adherent macrophages. This experimental model provides an opportunity to examine the effects of thermal injury upon essentially normal macrophages (those receiving the mild stimulus of ip saline) and upon the process of differentiation of macrophages to a more active state.

The results in this report indicate that thermal injury to the skin produces delayed, selective effects on the in vivo activation of peritoneal macrophages by Con A. Although the burn did not significantly decrease the number of macrophages recovered from the peritoneal cavity or alter the Con A-induced increase in phagocytic ability of glass-adherent macrophages, the other parameters of Con A-induced activation were significantly decreased. These observations indicate that the capacity of macrophages to respond to infection may be limited after thermal injury.

Previous studies indicate that host resistance to infection is altered after thermal injury (9, 16). Humoral immunity seems to be normal (3, 14), but cellular defense mechanisms are altered. In the first 3 days postburn, there are alterations in inflammation in the skin (10) and peritoneal cavity (2, 10), in the capacity of the RES to clear colloidal particles from the circulation (20, 21, 24), and in the ability of the animals to resist challenge with *Pseudomonas aeruginosa* (2, 9). Delayed effects also occur. Leukocyte enzymes, β-glucuronidase, lysozyme, and acid phosphatase, reach their lowest levels 6 to 10 days postburn (1). Decreased resistence to infection is found for some time postburn in humans, and intermittent or cyclic changes in leukocyte bactericidal activity have been seen several weeks postburn (4). Skin graft rejection is significantly prolonged in burned animals (15, 18) and humans (5, 8), and delayed hypersensitivity is depressed for up to 7 weeks postburn (19). The changes in macrophage activity observed in this report may be related to other delayed or prolonged effects of thermal injury.

Several studies suggest that alterations in macrophage activity are important in the decreased resistance to infection after thermal injury. Resistance to *P. aeruginosa* challenge could be increased in burned mice by transfer of peritoneal exudate cells rich in macrophages (13). Furthermore, the observations that graft rejection (18) and delayed hypersensitivity (19) are depressed are significant in this regard because these reactions involve macrophages. Because lymphocyte reactivity has not been found to be depressed in burned animals (6, 22) or humans (11), an understanding of the effects of burns on macrophages seems particularly important.

ACKNOWLEDGMENT

The technical assistance of Beth Rudloff is gratefully acknowledged.

This investigation was supported by Public Health Service grant FR 05656-04 from the Division of Research Facilities and Resources to the College of Human Medicine, Michigan State University.

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