Different Lymphocyte Compartments Respond Differently to Mitogenic Stimulation After Thermal Injury

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Because of the association between the development of an immunocompromised state and an increased risk of infection, increasing attention has been focused on describing and characterizing the immune consequences of thermal injury. Results of human studies are largely based on the *in vitro* responsiveness of peripheral blood leukocytes, while splenocytes are generally used in the animal studies. Because the response of lymphocytes from different lymphocyte compartments may vary, we compared the responses of murine peripheral blood, splenic, Peyer's patch, and mesenteric lymph node lymphocytes to a battery of mitogens after thermal injury. Burn-induced immunosuppression was maximal in the splenic lymphocyte compartment, where the responses to all three test mitogens were depressed throughout the 28-day postburn study period. Although the PHA-induced mitogen response of lymphocytes from the other three lymphoid compartments remained suppressed throughout the study period, the response to the mitogens Con-A and PWM generally returned to normal or supranormal levels by the seventh postburn day. Therefore it appears that the effect of a thermal injury on lymphocyte function varies according to the lymphocyte compartment examined and the mitogen tested. These results raise the question of whether animal studies using splenic lymphocytes can be correlated with human studies performed on circulating blood lymphocytes.

FAILURE OF THE immune system leading to an increased susceptibility to infection is a major cause of morbidity and death of the burn victim.[1] For this reason, during the past two decades, a tremendous effort has been given to investigating the mechanisms promoting burn-induced immune failure. These studies have used human burn victims and animal models. Most of the animal studies have been performed in rodents because the immune system of the rodent has been well characterized, large numbers of animals can be studied at a reasonable cost, and the technical demands of inflicting a thermal injury in the rodent are minimal. However one major difference between these animal and human studies, which has received little attention, is that conclusions on the effects of thermal injury on cell-mediated immunity are based on studies of peripheral circulating leukocytes in humans, while splenocytes are generally studied in the murine models. Yet the response of lymphocytes from anatomically different compartments to the same stimulus can differ.[2,3] Furthermore the responses of B and T cells to antigen *in vivo* take place largely in lymphoid tissue, not in the bloodstream.[3] Thus it is important to know whether the response of circulating peripheral lymphocytes to a thermal injury is similar to that seen in splenic lymphocytes. In addition, because gut barrier failure leading to bacterial translocation has been documented to cause systemic infection in burned mice and rats,[4–6] studies of intestinal immunity after thermal injury are also important. Therefore the goal of the present study was to measure and compare simultaneously the blastogenic response of peripheral blood, splenic, and gut-associated lymphocytes to a battery of mitogens in a murine burn model.

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Materials and Methods

Mice

Seven-to-10-week-old, specific pathogen-free, inbred Balb/c mice of both sexes weighing 24 g to 32 g were used in these experiments. Inbred Balb/c mice were chosen for study because variability between animals is reduced in inbred compared to outbed strains. The mice were housed under barrier-sustained conditions and maintained in accordance with the recommendations of the Guidelines

for the Care and Use of Laboratory Animals. The experiments were approved by the LSU-Medical Center Animal Care Committee.

Thermal Injury

The mice were burned according to the procedure described by Walker et al.[7] The mice were anesthetized with intraperitoneally administered sodium pentobarbital (0.4mg/10 g of body weight). The hair was removed from the back and the mouse was placed in a plastic holder containing a 2.5×3.5 cm^2 opening to allow exposure of the animal's back to the boiling water. The exposed back was immersed in boiling water for 5 seconds to produce a 25% total body surface area (TBSA) third-degree burn. The mice were immediately resuscitated with 2 mL of intraperitoneally (IP) administered sterile saline.

Cell Preparation

Mice were sacrificed 1, 3, 5, 7, 14, or 28 days after burn and peripheral blood, spleen, mesenteric lymph node, and Peyers patch mononuclear cells were isolated as follows. After the mice were anesthetized with IP sodium pentobarbital, the thoracic cavity was opened aseptically with sterile instruments. Approximately 1 mL of blood was collected in heparin (100 units) by cardiac puncture. An abdominal incision was then made and the spleen and the mesenteric lymph node complex were removed and placed in cold (4 C) minimal essential medium (MEM) supplemented with 2mM L-glutamine and containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and heat-inactivated fetal calf serum (2% v/v). After these organs were removed, the small intestine containing the Peyers patches was excised.

Peripheral blood mononuclear cells were isolated as follows. The blood was mixed with 1 mL of MEM supplemented with 2mM L-glutamine and containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and heat-inactivated fetal calf serum (2% v/v). The blood-modified MEM mixture was layered over 1.5 mL of ficol-hypaque and centrifuged for 15 minutes at 1200 to 1500 RPM. The mononuclear cell interface was collected and washed twice with modified MEM. The washed cell pellet was incubated in 1 mL of 0.14 M ammonium chloride for five minutes at 37 C to lyse contaminating erythrocytes. The mononuclear cells were then washed twice with modified MEM, counted, and resuspended in RPMI 1640 containing 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% v/v fetal calf serum at a final concentration of 4×10^6 cells per milliliter. Cell viability was more than 95% by trypan blue dye exclusion.

Splenic mononuclear cell populations were obtained by sieving the spleen through sterile 100-gauge stainless steel wire mesh. The single cell suspensions were washed twice with modified MEM and adjusted to a final concentration of 4×10^6 cells/mL in RPMI 1640 containing 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% v/v fetal calf serum. Viability was verified by trypan blue dye exclusion.

The mesenteric lymph nodes were gently minced and then passed through three layers of sterile rayon/polyester gauze to create a single-cell suspension. The single-cell suspension was washed three times with modified MEM and adjusted to a final concentration of 4×10^6 cells/mL in RPMI 1640 containing 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% v/v fetal calf serum. Viability was verified by trypan blue dye exclusion.

The Peyers patches were excised from the serosal side of the intestine with care being taken not to enter the lumen. The Peyers patches were then teased apart with sterile forceps and further fragmented by gentle pipetting. Approximately five to seven Peyers patches were obtained from each animal. The fragments were treated with type 1 collagenase (50 U/mL) in modified MEM for 60 minutes at 37 C.[8] After collagenase digestion, the cell suspension was passed through three layers of sterile rayon/polyester gauze and subsequently washed three times in modified MEM. The cell suspension was adjusted to a final concentration of 4×10^6 cells/mL in RPMI 1640 containing 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% v/v fetal calf serum. Viability was verified by trypan blue dye exclusion.

Mononuclear cell preparations from the blood, spleen, mesenteric lymph nodes, or Peyers patches were placed into microtiter plates. The blastogenic responses of these cell cultures (2×10^5 cells) were tested using the following mitogens: phytohemagglutinin (PHA) (1 μg), concanavalin A (Con-A) (2.5 μg), or pokeweed mitogen (PWM) (5 μg). PHA and Con-A are primarily T-cell mitogens and PWM is primarily a B-cell mitogen, although none of these agents affect one cell population exclusively.[9] Simultaneous cell cultures were run in the absence of mitogen stimulation. The cells were incubated for 72 hours in 5% CO_2 at 37 C, pulsed with 0.5 uCi of tritiated thymidine, and incubated an additional 18 hours. The cells were harvested with a cell harvester and counted in a scintillation counter. Each assay was run in triplicate and the results were averaged and the mean value was used for statistical analysis.

Statistics

Comparisons between groups were analyzed using analysis of variance (ANOVA) with the Newman-Keuls test. Statistical significance was considered to be achieved at $p < 0.05$.

Results

Table 1 illustrates the blastogenic response of blood, splenic, mesenteric lymph node (MLN), and Peyers patch lymphocytes to the three test mitogens. The blastogenic responses of lymphocytes from these four lymphocytes compartments varied in their ability to respond to the different mitogens and in the magnitude of their responses. The most responsive lymphocyte population was the splenic lymphocytes, followed by the MLN, blood, and Peyers patch lymphocytes. Of the mitogens tested, Con-A induced the greatest blastogenic response by all four lymphocyte populations. These results indicate that peripheral blood lymphocytes do not respond as well to mitogens (at the doses tested) as splenic or MLN lymphocytes. Although the blastogenic response varied among the lymphocyte populations, the level of tritiated thymidine uptake by unstimulated cells was similar among all four lymphocyte populations.

As described by others,[10,11] the response of splenic lymphocytes to mitogens was severely depressed after a thermal injury (Fig. 1). Three days after burn, the blastogenic response of the splenic lymphocytes to all three mitogens was significantly depressed. Although the blastogenic response of the splenic lymphocytes was returning toward normal, it remained significantly depressed even 28 days after burn. The pattern of spontaneous thymidine incorporation by the splenic lymphocytes differed from the mitogen-stimulated response. Spontaneous blastogenesis was decreased three and five days after burn, however, in contrast to mitogen-stimulated blastogenesis, spontaneous blastogenesis, which was significantly increased seven and 14 days after burn.

The blastogenic response of the peripheral blood lymphocyte population differed from the splenic population in several ways. First spontaneous blastogenesis of the circulating lymphocytes was increased by day 3 after burn and remained increased through day 14 after burn (Fig. 2). Second although the PHA response of the circulating lymphocyte population remained depressed throughout the 28-day postburn period, and thus was similar to the splenic response, the response of the circulating blood lymphocytes to Con-A and PWM differed from that found in the splenic population. The suppressive effect of a thermal injury on the blastogenic response to Con-A and PWM was shorter lived in the peripheral blood lymphocytes than in the splenic lymphocytes. Seven days after burn the circulating lymphocyte response to Con-A or PWM was normal or increased and remained normal throughout the study period. Thus the spontaneous and stimulated blastogenic responses after thermal injury were not identical between circulating and splenic lymphocytes.

The pattern of mitogen-stimulated blastogenesis of the MLN lymphocyte population was similar to the pattern found in the peripheral blood lymphocytes (Fig. 3). The PHA response remained depressed for the 28-day study period, while the burn-induced depression of lymphocyte blastogenesis to Con-A or PWM was complete by the seventh postburn day. The spontaneous blastogenic response of the MLN lymphocytes was normal at all time points.

The pattern of mitogen-stimulated blastogenesis of the Peyers patch lymphocytes was similar to that found in the peripheral blood lymphocytes, with the exception that the response to Con-A or PWM was greater than normal on day 28 postburn (Fig. 4). The pattern of spontaneous blastogenesis was similar to that found in the spleen with an early decrease in blastogenesis followed by increased levels of thymidine incorporation.

Discussion

The major finding of this murine study is that the effect of a thermal injury on lymphocyte function varies based on the lymphocyte compartment examined, as well as on the mitogen tested. Thus our results are consistent with the recent experimental studies of Burleson et al.[12] on the effects of a thermal injury on lymphocyte subpopulation levels. These authors found that the ratio of T-helper to T-suppressor/cytotoxic lymphocytes from blood, spleen, and lymph nodes of burned and burned-infected rats varied based on the lymphocyte compartment measured. Although measurements were made only at one time (two days after burn), it was clear that lymphocyte subpopulation changes after burn and infection were different for each tissue tested.[12] The effect of thermal injury on different lymphocyte compartments in humans has not been studied; however the blastogenic response of peripheral blood lymphocytes to different mitogens has been shown to vary in humans sustaining thermal injuries.[13,14]

Although *in vitro* assays of mononuclear cell function and activity were developed to study cell-mediated immunity, use of these assays to elucidate the immune state

TABLE 1. *Comparison of Mitogenic Response Between Mononuclear Cell Populations Isolated from Different Lymphocyte Compartments*

Mononuclear Cell Source	n	Media	PHA	Con-A	PWM
Blood	14	277 ± 85	3002 ± 707	6617 ± 1634	2637 ± 583
Spleen	14	305 ± 51	6387 ± 1420	68,567 ± 10,851	15,034 ± 3916
MLN	14	324 ± 93	4278 ± 720	30,548 ± 5877	5181 ± 689
Peyers patches	14	270 ± 64	502 ± 84	2613 ± 877	634 ± 125

Data expressed as mean ± SD tritiated thymidine uptake in disintigrations per minute (DPM).

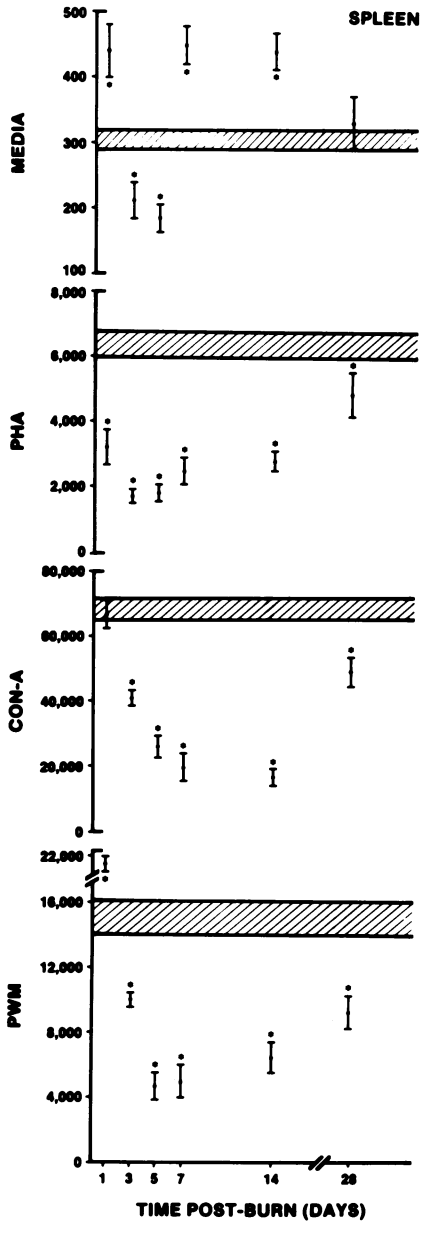

FIG. 1. Response of unstimulated and mitogen-stimulated (PHA, CON-A, PWM) splenic lymphocytes harvested from 1 to 28 days after thermal injury. All values expressed as mean ± SEM DPMs of tritiated thymidine uptake. Hatched bar represents control lymphocyte incorporation of tritiated thymidine (mean ± 1 SEM).

* Indicates that experimental value is different ($p < 0.05$) from control value by ANOVA.

of the burn patient has been confusing. Using the blastogenic response to PHA as an example, some authors have reported an increased mitogenic response in burn victims,[15,16] while others have reported a blunted[17-19] or normal mitogenic response[20] in burn victims. In addition some investigators have reported that the cellular response to PHA correlates with sepsis and/or death[21,22] while others have not.[17,20,23] Part of the difference between these previously reported studies may be related to differences in mononuclear cell preparation, techniques of performing the *in vitro* mitogen assay, or patient selection. In fact recent human[24-26] and animal[27] studies have documented that density gradient purified peripheral blood mononuclear cells from burn victims but not normal controls are contaminated with many nonlymphoid cells. Furthermore recently we have demonstrated that the presence of these contaminating leukocytes causes artifactual alterations in standard *in vitro* tests of lymphocyte function.[24] These human studies emphasize the difficulties of assessing the global systemic immune state of burn victims based on measurements of just one lymphocyte compartment.

In contrast to these human studies, the splenic response

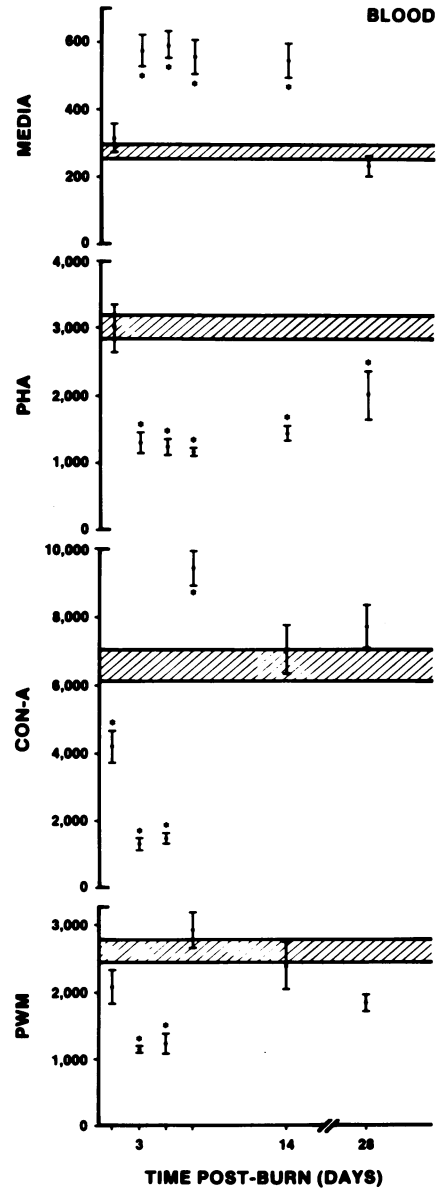

FIG. 2. Response of unstimulated and mitogen-stimulated peripheral blood lymphocytes. See Figure 1 legend for details.

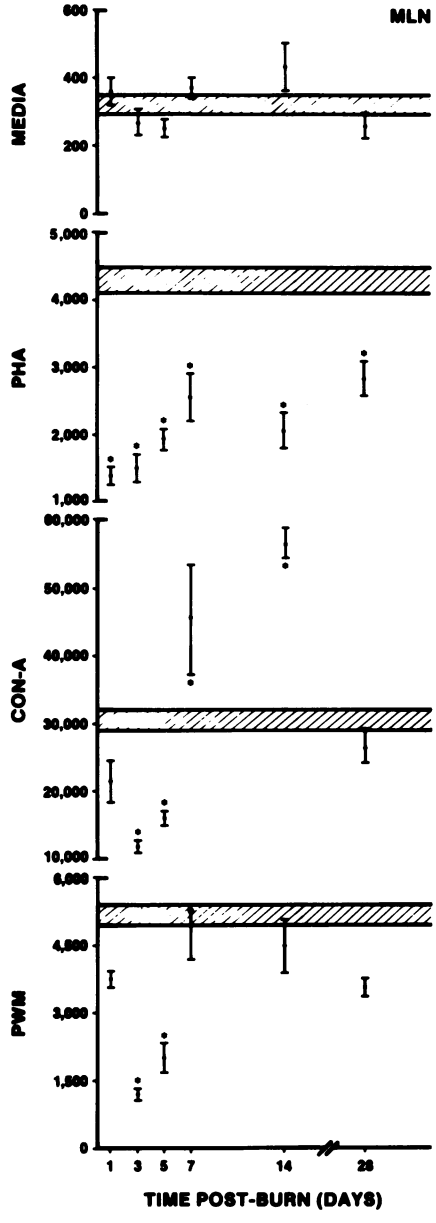

FIG. 3. Response of unstimulated and mitogen-stimulated mesenteric lymph node lymphocytes. See Figure 1 legend for details.

to different soluable and cellular blastogenic stimuli has been an accurate predictor of global immune competence after thermal injury in animals.[28,29] One explanation for this fact may be that the splenic response to stimuli more closely reflects the immune state of the burned animal than the response of other lymphocyte compartments. This explanation is supported, at least in part, by our results documenting that of all the lymphocyte compartments tested, the blastogenic response of splenic mononuclear cells was the most consistently impaired.

With the development of recombinant DNA technology, it has become possible to produce large quantities of purified endogenous biologic response modifiers, such as IL-1, IL-2, gamma-Interferon, and colony-stimulating factors. Many of these factors have been tested in murine burn models and shown to improve survival and bolster various arms of the immune system, especially the response of splenic mononuclear cells.[30–32] Thus it is possible that these biologic response modifiers may be effective in improving host immune defenses in burn victims and thereby improve survival. However, because in humans only peripheral blood lymphocytes can be sampled, and the mitogenic response of peripheral blood lymphocytes may not fully reflect the immune state, it may be difficult to monitor the immunomodulating effects of these agents in clinical practice. Our finding that the blastogenic response of peripheral blood and splenic lymphocyte populations may vary demonstrates the potential difficulty of

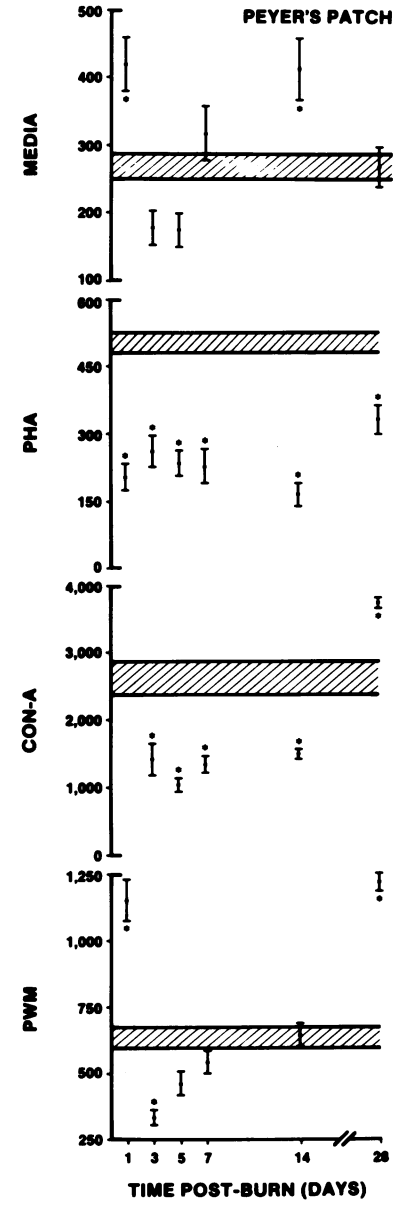

FIG. 4. Response of unstimulated and mitogen-stimulated Peyer's patch lymphocytes. See Figure 1 legend for details.

accurately monitoring immune competence by measuring just the response of peripheral blood lymphocytes.

Because intestinal antibacterial barrier function fails after thermal injury, resulting in the translocation of intestinal bacteria to systemic organs,[4,5] we also measured the effect of a thermal injury on intestinal immunity. To our knowledge these are the first studies to investigate whether the response of Peyers patch or MLN lymphocytes to mitogens are altered after thermal injury. The results of these studies indicate that the blastogenic response of Peyers patch and MLN lymphocytes are impaired after thermal injury. The overall patterns of response of lymphocytes from these two lymphocyte compartments were similar but not identical. In both compartments the blastogenic response to Con-A and PWM returned to normal by 28 days after burn, while the blastogenic response to PHA remained depressed throughout the study period. Thus the mitogenic responses of Peyers patch and MLN lymphocytes after thermal injury were similar to the general response of peripheral blood lymphocytes and different from splenocytes. The clinical significance of these findings are speculative. However the fact that intestinal immunity is abnormal after a nonlethal thermal injury is consistent with our previous studies documenting that thermal injury promotes bacterial translocation in Balb/c mice.[33]

These studies demonstrate the compartmentalized response of different lymphoid tissues to mitogens after thermal injury. Further studies are needed to determine the potential biologic and clinical significance of these findings.

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