Changes in Lymphocyte Number and Phenotype in Seven Lymphoid Compartments After Thermal Injury

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Thermal injury is associated with dysfunction of host defense systems. The present study used flow cytometric immunofluorescence analyses to investigate changes in number and phenotype of lymphocytes in seven different lymphoid compartments at 2, 6, 12, 24, 48, and 60 days after 50% total body-surface area thermal injury in the rat. Relative to sham-injured control rats, at postburn day 2, significant lymphopenia was observed in the peripheral blood along with depletion of lymphocytes from the spleen and thymus. By day 6 after injury, lymphocytes in the bone marrow and cervical lymph nodes decreased significantly while numbers in the spleen and thymus remained depressed. Splenic and cervical node lymphocyte numbers normalized by day 12, the bone marrow and thymus numbers still were significantly lower than control, and a 6.5-fold increase in number of lymphocytes was observed in the nodes draining the burn wound, pooled axillary, brachial, inguinal, and lumbar lymph nodes. At day 24 after injury, the thymus and bone marrow virtually were depleted of lymphocytes, the mesenteric lymph nodes manifested a significant decrease, and lymphocytes in the nodes draining the burn wound continued to increase in number. This same pattern was maintained on day 48, but numbers of lymphocytes in the mesenteric nodes normalized. At day 60 after injury, lymphocyte numbers in all tissues were normalized, but the spleen and nodes draining the burn wound where increased numbers compared to control persisted. Cell-surface phenotyping was performed on all lymphoid tissues at all time intervals to determine the percentages of lymphocytes comprising the following subsets: Ia+ cells (B cells and activated T cells), T cells, $T_{Helper/Inducer}$ cells ($T_{H/I}$), and $T_{Suppressor/Cytotoxic}$ ($T_{S/C}$) cells. Although changes in lymphocyte subset percentages were complex, they could be divided grossly into two phases. First, all compartments showed significant phenotypic changes in the first six days after burn. With the exception of the nodes draining the burn wound and the blood, this was followed by a return towards normal on day 12. The second phase then ensued with significant phenotypic changes again occuring in most tissues from days 24 to 60 after injury. These studies demonstrate that burn injury results in dramatic alterations in lymphocyte numbers and subset percentages in different lymphoid compartments. Immune alterations observed following thermal injury may be due, in part, to a redistribution of the cellular elements responsible for generation of the immune response.

DURING ADULT LIFE, a disperse anatomic system provides the spatial organization for the specific immune system. This consists of (1) the bone marrow and thymus, i.e., lymphopoetic organs; (2) the primary lymph nodes, i.e., sites of collection of mature lymphocytes where regional immune responses occur; (3) injured or inflammatory tissues, i.e., sites of collections of mature lymphocytes where localized immune responses occur; (4) the spleen and secondary lymph nodes, i.e., sites of collections of mature lymphocytes where systemic immune responses are amplified; and (5) the blood and lymphatics, i.e., transport systems to move lymphocytes among these various anatomic sites. It has been established[1-3] that lymphocytes continually recirculate through these lymphoid tissues, spending variable amounts of time in the different compartments, and that the duration of time spent in any compartment is a function of the dynamic interplay of immunologic stimuli present. Therefore, a successful immune response depends on the movement of lymphocytes to the appropriate anatomic site(s)[4-6] as well as the temporal sequence of regulatory and effector lymphocyte generation.[7,8] On this basis, the immune response can be viewed as being comprised of both spatial and temporal components. It would follow that dysfunction of either or both of these components would be deleterious for the development of an appropriate immune response.

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Dysfunction of specific immunity is a consequence of thermal injury, and it generally is accepted that this contributes importantly to increased susceptibility to infection. Cell-mediated immune manifestations of thermal injury include (1) skin test anergy to primary and recall antigenic stimulation[9,10]; (2) depression of lymphocyte proliferation in response to T-cell mitogens and allogeneic and autologous cells[11-13]; (3) decreased activity in natural killer and antibody-dependent cellular cytotoxicity assays[14]; and (4) increased allograft rejection times.[15] There are conflicting data concerning the effect of thermal injury on humoral immunity. Although serum immunoglobulin levels decrease markedly in the immediate postburn period,[16,17] production of hemolytic antibodies to specific antigens is reportedly enhanced[18] or decreased.[19]

Although these thermal injury-induced immune defects have been documented repeatedly, most studies identifying these abnormalities have been limited to investigation of a single tissue or lymphoid compartment such as the blood, skin, or blister fluid in humans or blood, skin, and spleen in animals. While these reports have yielded considerable useful information, caution should be exercised in assessing whole-body immune status on the basis of a single tissue such as the spleen, skin, or peripheral blood[20,21] because, as described above, the immune system is anatomically dispersive in organization. Thus, evaluation of immune capacity in one anatomical site may, in some cases, bear little relationship to that in a distant anatomical site. With this in mind, the present study was performed to determine numerical and phenotypic lymphocyte changes in seven different lymphoid compartments as a function of time postburn in the thermally injured rat.

Materials and Methods

Animals

Male Lewis rats (325 to 400 g) were obtained from Charles River Laboratories (Wilmington, MA). Rats were kept grouped in hanging cages for two weeks to exclude the presence of underlying diseases. Following thermal injury, animals were housed individually in light- (L:D, 12:12) and temperature- (23 C) controlled colony rooms and allowed food and water *ad libitum*. Rats were treated and maintained according to the NIH Guide for the Care and Use of Laboratory Animals.

Thermal Injury

Animals were anesthetized with pentobarbital and subjected to a 50% total body surface area (25% full-thickness dorsal and 25% full-thickness ventral) scald injury using the standard Walker-Mason model.[22] Lactated Ringer's solution (30 ml) was administered intraperitoneally before burning the ventral surface to serve as both a resuscitation fluid and to protect the intra-abdominal organs from injury. Sham-injured animals were treated identically except for exposure to 37 C water instead of 100 C water.

Experimental Design

At 2, 6, 12, 24, 48, and 60 days after injury, blood was obtained by cardiac puncture under ether anesthesia. Animals were decapitated, and the following tissues were harvested: spleen, thymus, both femurs, cervical lymph nodes, mesenteric lymph nodes, and the nodes that drain the burn wound (NDB; a pooled population of axillary, brachial, inguinal, and lumbar nodes). These tissues were then evaluated for weight, lymphocyte recovery, and cell-surface phenotype (see below). Body weights were not quantified at time of sacrifice, but other similarly burned animals in this laboratory have manifested a maximum of 4% weight loss at up to 24 days after injury.[23] Groups at each time point consisted of ten randomly selected animals, except for the postburn day 2 group, which was comprised of seven randomly selected rats. Sham-injured controls were evaluated two days after sham injury. No significant changes in lymphoid organ weight, total lymphocyte numbers, or phenotype percentages were observed between day 2 postsham-injured animals and normal uninjured rats. Because of this, concurrent sham-injured rats were not evaluated at postburn days 6, 12, 24, 48, and 60.

Tissue Preparation

The tissues were placed in cold Hank's Balanced Salts Solution without Ca^{++} or Mg^{++} (HBSS), and, except femurs and blood, were weighed to the nearest milligram on an electronic balance. For spleen, thymus, and nodes, single-cell suspensions were generated by gentle dispersion with a loose-fitting teflon homogenizer. Connective tissue and other debris were removed by sedimentation at unit gravity, erythrocytes were lysed with bicarbonate-buffered NH_4Cl solution, and leukocytes were washed twice with cold HBSS. Bone marrow suspensions were obtained by fracturing each femur and extruding the contents with a 25-gauge needle fitted to a syringe containing HBSS. This mixture was repeatedly aspirated into and expelled from the syringe until a suspension was generated. Red blood cells were then lysed and leukocytes were washed as above. Blood was diluted 1:2 with HBSS, layered on Ficoll-Hypaque, and centrifuged at 450 × g for 30 minutes. Mononuclear cells were harvested from the interface and were washed twice with HBSS.

Quantification of Lymphocytes

An aliquot of cells from each tissue suspension was removed, diluted appropriately, and a fixed volume was

TABLE 1. *Monoclonal Antibodies Employed and Lymphocyte Subsets Identified*

Clone	Lymphocyte Subset Identified*	CD# or Antigen
OX-6	B and activated T	Ia, nonpolymorphic
W3/13	T	Sialoglycoprotein
W3/25	$T_{Helper/Inducer}$ ($T_{H/I}$)	CD4
OX-8	$T_{Suppressor/Cytotoxic}$ ($T_{S/C}$)	CD8

* Subsets identified by immunofluorescence flow cytometric analysis of scatter-gated (forward × right angle) lymphocytes.

aspirated into a Spectrum III flow cytometer (Ortho Diagnostic Systems, Westwood, MA) for determination of quantitative lymphocyte count by light scatter analysis. The lymphocyte cluster was surrounded by an elliptical scatter gate in dot plot mode on a 2140 data analysis system (Ortho Diagnostic Systems, Westwood, MA) using simultaneous forward- *versus* right-angle light scatter intensities. Proper setting of the scatter gate was assessed by inspection of the individual forward- and right-angle scatter histograms of cells falling within the gate and adjusting the gate until both histograms were gaussian in appearance. The number of lymphocytes in this scatter gate were then determined. Because the Spectrum III flow cytometer counts all cells in a fixed volume of solution (rather than a preset number of cells or for a preset period of time), it then was possible to convert the number of lymphocytes in the counting region to a quantitive recovery of lymphocytes from the tissue under analysis. Of course, recoveries of cells were not "absolute" because each tissue suspension was washed before analysis. However, all tissue and blood samples were treated identically, and it is assumed that losses incurred during preparation were relatively constant.

Cell-surface Phenotype Analysis

After determination of lymphocyte counts, aliquots of 1×10^6 lymphocytes from each tissue were distributed to tubes for immunofluorescence analyses. To each tube was added a saturating amount of one of the primary monoclonal antibodies as listed in Table 1 (all antibodies were from Accurate Scientific, Westbury, NY). Autofluorescence controls received either mouse IgG or no primary antibody. Tubes were incubated on ice for 30 minutes and then were washed twice with phosphate-buffered saline containing 1% bovine serum albumin and 0.1% NaN_3 (PBS-BSA). A 1:50 dilution of fluorescein isothiocyanate-$(Fab')_2$ sheep antimouse IgG (this antibody showed no reactivity with rat IgG) was then added to all tubes. Another 30 minute incubation on ice was performed, the samples were washed twice in PBS-BSA, and suspended in PBS for fluorescence flow cytometry.

For fluorescence analyses, elliptical scatter gates were set on the lymphocyte cluster as described above for assessment of lymphocyte recovery. In the green fluorescence histogram mode, two mutually exclusive counting regions were set so that 98.5% to 99.5% of autofluorescence control cells fell in region 1. Lymphocytes in positively stained samples exhibiting fluorescence greater than region 1 were considered "fluorescence-positive." Results were obtained as the percentage of fluorescence-positive lymphocytes relative to total lymphocytes counted, usually 6000 to 10,000. For determination of numbers of fluorescence-positive lymphocytes, this percentage, expressed as a decimal, was multiplied by the lymphocyte recovery obtained as described above. As a means of assessing within assay variability, lymph-node and splenic cells from a single normal control rat were harvested, and replicate aliquots of these cells were labeled with monoclonal antibodies for fluorescence flow cytometric analysis. Intra-assay percentage coefficients of variation (standard deviation/mean × 100) for normal control lymph node lymphocytes were 6.6%, 0.8%, 1.7%, and 2.5% for the Ia, T, $T_{H/I}$, and $T_{S/C}$ markers, respectively. Intra-assay percentage coefficients of variation for normal control spleen lymphocytes were 6.6%, 1.5%, 2.2%, and 4.1% for the Ia, T, $T_{H/I}$, and $T_{S/C}$ markers, respectively.

Data Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with time after burn as the independent variable; sham-injured rats were assumed to be time after burn of 0. Separate ANOVAs were performed for each dependent variable, i.e., %W3/13+ lymphocytes, %W3/25+ lymphocytes, #OX−8+ lymphocytes, and so on. If a given ANOVA was significant at $p < 0.05$, Dunnett's Test was performed to compare the means of the various time points to the mean of the sham group.

Results

Tissue Weight (Table 2)

Relative to sham-injured rats, mean splenic weight decreased slightly at postburn day (PBD) 2, but by PBDs 6 and 12, mean splenic weight had returned to approximately control values. However, by PBD 24, splenic weight had increased 3.3-fold ($p < 0.01$) over that of sham-injured rats and remained significantly increased on PBD 48. Finally on PBD 60, splenic weight had returned to a value not significantly different than that of control animals.

Like splenic weight, thymus weight decreased slightly relative to control on PBD 2, but unlike the spleen, the thymus continued to decrease in weight on PBDs 6 and 12 ($p < 0.01$ *vs.* sham). Thymic weight increased markedly to 238 mg at PBD 24, representing a 1.4-fold increase compared to control ($p < 0.05$). The weight of the thymus

TABLE 2. *Effect of Time Post-Burn on Lymphoid Tissue Weight*

Lymphoid Tissue	Sham	Time Post-Burn					
		2 Days	6 Days	12 Days	24 Days	48 Days	60 Days‖
Spleen	710 ± 20‡	560 ± 40	890 ± 60	950 ± 40	2,346 ± 320†	1293 ± 230†	944 ± 170
Thymus	170 ± 10	132 ± 30	86 ± 10†	80 ± 10†	238 ± 40*	279 ± 30†	282 ± 20†
Cervical nodes	146 ± 10	84 ± 30	130 ± 40	156 ± 50	338 ± 100†	445 ± 140†	210 ± 100
Mesenteric nodes	125 ± 30	100 ± 30	144 ± 15	113 ± 40	126 ± 40	121 ± 40	144 ± 60
Nodes draining burn wound‖	170 ± 20	174 ± 10	355 ± 40	721 ± 40†	621 ± 50†	595 ± 60†	653 ± 90†

* $p < 0.05$.

† $p < 0.01$ *versus* sham-injured control by analysis of variance and Dunnett's test.

‡ Numbers represent the mean ± SEM tissue weight in milligrams.

‖ Nodes draining the burn wound: axillary brachial, inguinal, and lumbar nodes.

then remained significantly elevated throughout the remainder of the postburn course.

The pattern of change in cervical lymph-node weight was fairly similar to that of the spleen. Thus, cervical lymph-node weight decreased at PBD 2, returned to control levels at PBD 6, and then remained unchanged at PBD 12. By PBD 24, cervical node weight had approximately doubled relative to control ($p < 0.01$). This increase continued at PBD 48, but by PBD 60 the mean weight of this tissue had returned to normal.

Mesenteric lymph-node weight displayed no significant change throughout the entire postburn course, the only compartment studied to do so.

The nodes draining the burn wound (NDB), again defined as pooled axillary, brachial, inguinal, and lumbar nodes, demonstrated no fluctuation in weight at PBD 2. However, NDB weight increased at PBD 6 and was elevated 6.5-fold ($p < 0.01$) by PBD 12. The weight of the NDB then remained significantly increased throughout the rest of the period of study.

Tissue Lymphocyte and Lymphocyte Subset Recovery

These data represent numbers of small resting lymphocytes unless otherwise indicated. Total lymphocyte recoveries are presented in Table 3, and also are plotted in Figures 1 and 2 (open circles) in order to compare directly total lymphocyte changes with changes in numbers of lymphocytes comprising the various subsets. Data from PBD 60 were excluded from the statistical analyses. This was thought to be justified because of the large variability in this group relative to all others; including the day 60 group in the analyses thus would have violated the assumption of homogeneity of variance among groups. However, data from PBD 60 are presented to demonstrate any trends that might have been present late in the postburn course.

Total lymphocyte number (TLN) in the spleen decreased significantly at PBDs 2 and 6 and then returned to levels not statistically different from normal for the remainder of the postburn course (Table 3, Fig. 1A). Numbers of lymphocytes comprising the four subsets evaluated (Ia+, pan T+, T helper-inducer, and T suppressor-cytotoxic) generally paralleled the changes in total lymphocyte number (Table 3, Fig. 1A), although there were significant changes in the relationships among the subsets. These relative changes are more parsimoniously presented as percentages of lymphocytes comprising the four subsets (see section below).

TABLE 3. *Effect of Time Post-Burn on Lymphocyte Recovery from Rat Lymphoid Tissues*

Lymphoid Tissue	Sham	Time Post-Burn					
		2 Days	6 Days	12 Days	24 Days	48 Days	60 Days#
Spleen	123 ± 7‡	69 ± 16†	86 ± 7*	107 ± 8	145 ± 2	146 ± 16	200 ± 65
Thymus	98 ± 2	37 ± 10*	14 ± 5†	26 ± 8†	2 ± 0.41†	6 ± 1.5†	125 ± 64
Bone marrow	22 ± 2	24 ± 2	5 ± 1†	4 ± 0.4†	3 ± 0.8†	15 ± 2*	23 ± 6.2
Cervical nodes	17 ± 3	11 ± 2	5 ± 1*	11 ± 2	19 ± 2.5	34 ± 5†	33 ± 33
Mesenteric nodes	42 ± 11	27 ± 3	30 ± 6	22 ± 8	8 ± 1*	26 ± 10	48 ± 29
Nodes draining burn wound‖	16 ± 3	9 ± 1	49 ± 13	106 ± 7†	79 ± 15†	111 ± 16†	184 ± 27
Blood	3506 ± 419¶	1256 ± 140†	3840 ± 357	537 ± 32†	2210 ± 91*	—	—

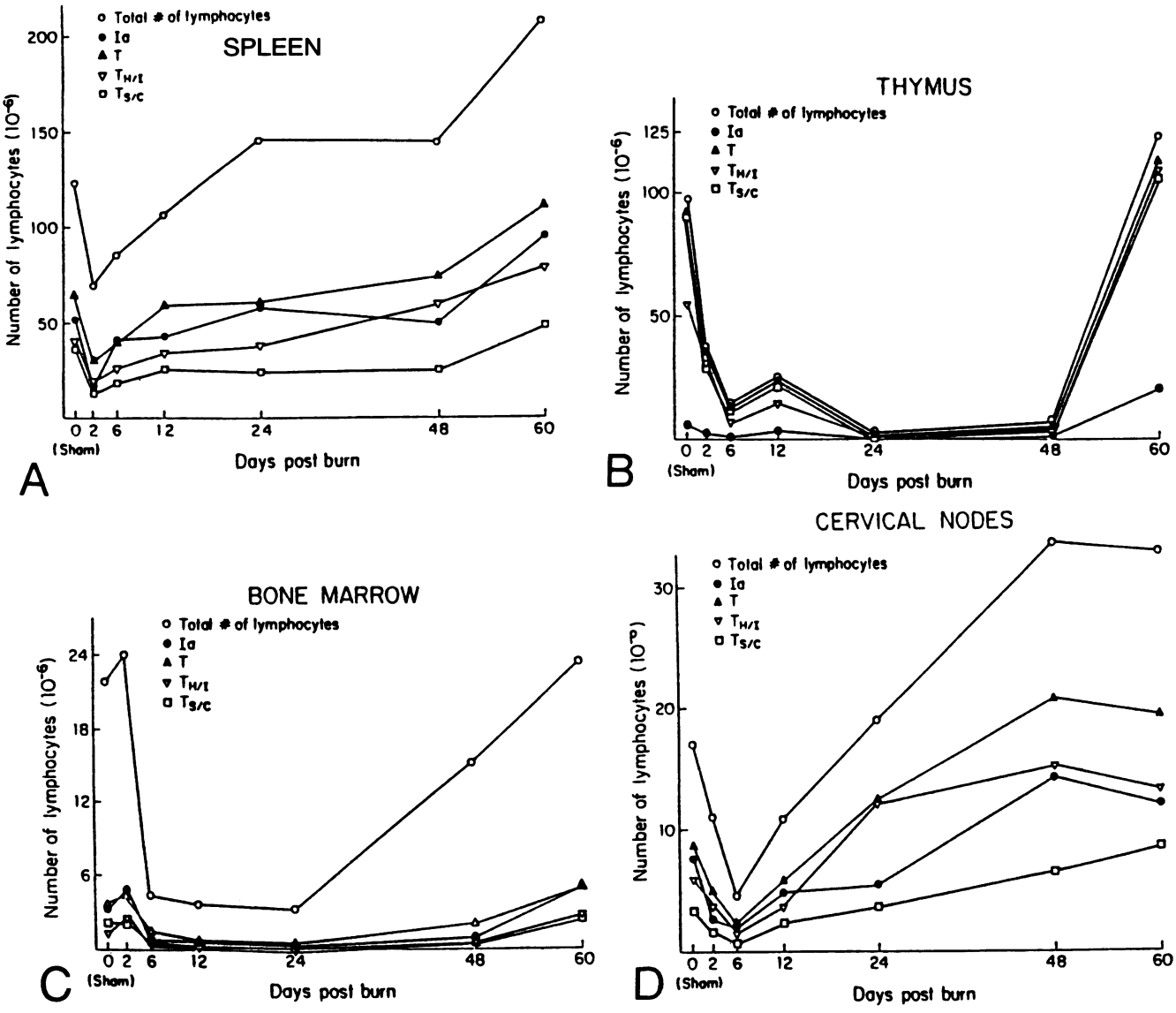
* $p < 0.05$, †$p < 0.01$ *versus* sham by analysis of variance and Dunnett's Test.

‡ Mean ± SEM × 10^{-6} lymphocytes per tissue.

‖ Nodes draining the burn wound: axillary, brachial, inguinal, and lumbar nodes.

¶ Mean ± SEM small lymphocytes/mm^3.

60 day PB data was not included in statistical analysis due to the large variability associated with this group that did not satisfy the assumption of homogeneity of variance (note large SEM at PBD 60). These data are included for purpose of comparison.

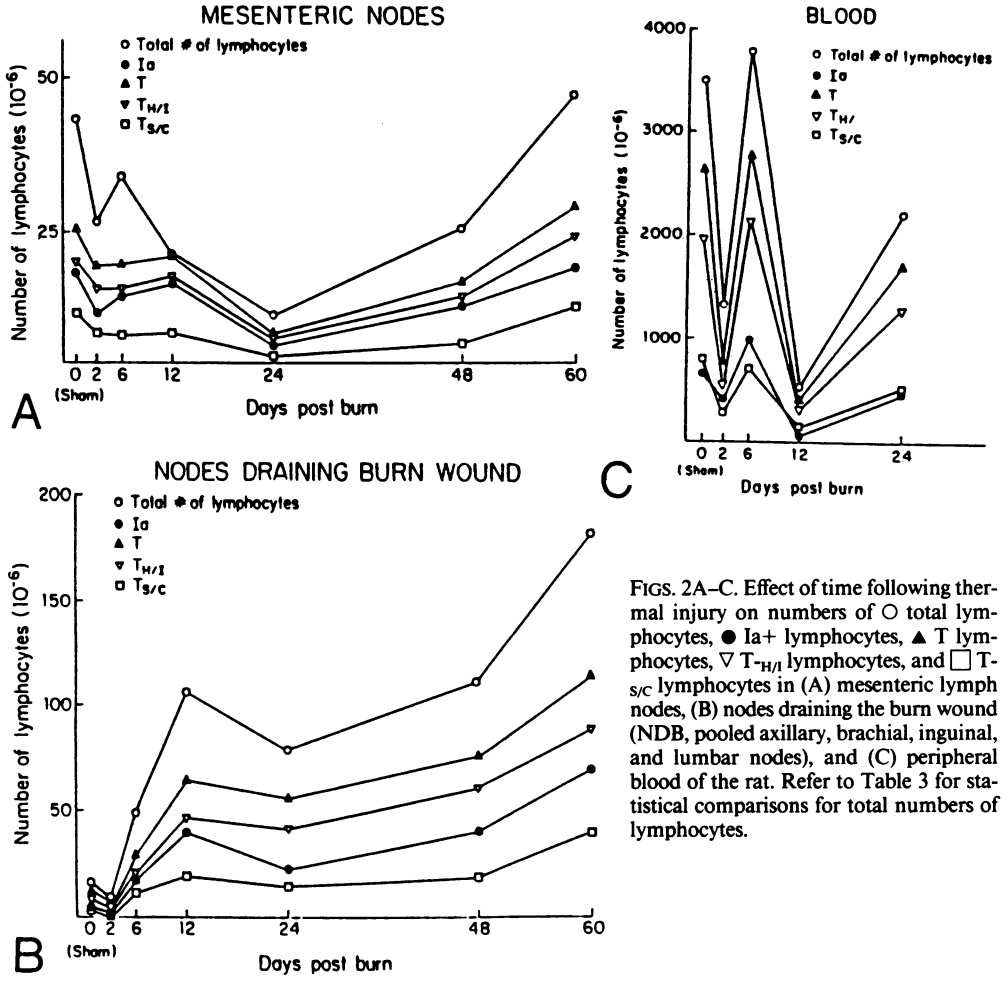


FIGS. 1A–D. Effect of time after thermal injury on numbers of O total lymphocytes, ● Ia+ lymphocytes, ▲ T lymphocytes, ▽ $T_{H/I}$ lymphocytes, and □ $T_{S/C}$ lymphocytes in (A) spleen, (B) thymus, (C) bone marrow, and (D) cervical lymph nodes of the rat. Refer to Table 3 for statistical comparisons for total numbers of lymphocytes.

Thymic TLN also decreased early in the postburn period (Table 3, Fig. 1B), but unlike the spleen, TLN decreased further at days 24 and 48. In fact, at these times thymic involution was almost complete with this tissue containing only 2×10^6 and 6×10^6 lymphocytes at days 24 and 48, respectively. However, between days 48 and 60 after burn, thymic repletion appeared to occur. As with the spleen, numbers of lymphocytes within the four subsets were dominated by changes in TLN (Table 3, Fig. 1B).

Bone marrow lymphocyte number increased slightly at PBD 2 (Table 3, Fig. 1C); this was the only tissue in which a substantial decrease was not observed at this particular time after burn. However by PBD 6, marrow TLN had decreased by 77% relative to control and remained at a low level until PBD 48, at which time TLN showed recovery towards normal. On PBD 60, mean TLN was almost identical to sham-injured controls. Note that while subset changes again appeared to parallel TLN (Table 3, Fig. 1C), the sum of the numbers of lymphocytes comprising the four subsets was far less than TLN. The reason for this is not known, but it is hypothesized that a majority of bone marrow lymphocytes are immature forms that may not express either Ia, W3/13, CD4, or CD8 markers.

TLN in the cervical lymph nodes decreased on PBD 2 (Table 3, Fig. 1D) and further declined on PBD 6 ($p < 0.05$). Thereafter, cervical node TLNs began to increase and by PBD 48 were elevated 2-fold over control (p



FIGS. 2A–C. Effect of time following thermal injury on numbers of ○ total lymphocytes, ● Ia+ lymphocytes, ▲ T lymphocytes, ▽ $T_{H/I}$ lymphocytes, and □ $T_{S/C}$ lymphocytes in (A) mesenteric lymph nodes, (B) nodes draining the burn wound (NDB, pooled axillary, brachial, inguinal, and lumbar nodes), and (C) peripheral blood of the rat. Refer to Table 3 for statistical comparisons for total numbers of lymphocytes.

< 0.01). At PBD 60, lymphocyte numbers still were increased by approximately a factor of two. As did TLN, lymphocytes comprising the four subsets showed a decline in the cervical nodes at PBDs 2 and 6 and began to recover by PBD 12 (Table 3, Fig. 1D). But between PBDs 12 and 24, helper-inducer T cells increased at a far more rapid rate than did Ia+ lymphocytes or suppressor-cytotoxic T cells. In contrast, from days 24 to 48 this situation was exactly reversed with Ia+ lymphocytes increasing more rapidly than helper-inducer T cells.

Changes in mesenteric lymph node TLN were distinct from those of any other tissue studied (Table 3, Fig. 2A). Instead of an abrupt decline at PBDs 2 to 6 followed by recovery of cell numbers as seen in the spleen and cervical nodes, lymphocytes in the mesenteric nodes declined fairly steadily until PBD 24 ($p < 0.05$ *vs.* sham), at which time numbers of these cells steadily increased to reach control levels by PBD 60. Lymphocyte subset changes appeared to occur in concert with changes in TLNs.

While all other tissues showed significant declines in TLN, the nodes draining the burn wound did not manifest a statistical decrease in TLN at any postburn time (Table 3, Fig. 2B). As with the cervical nodes, TLN in the NDB increased over control values, albeit with a slightly faster time course and with much greater magnitude than was observed for the cervical nodes. By PBD 12, TLN in the NDB had increased to 6.5-fold over control ($p < 0.01$ *vs.* sham), and numbers remained extremely elevated to the PBD 60 time point. Changes in numbers of lymphocytes comprising the four subsets once again largely changed in parallel with TLNs.

Numerical lymphocyte changes in the peripheral blood were only evaluated until PBD 24 (Table 3, Fig. 2C). TLNs decreased significantly ($p < 0.01$) on PBD two, returned to normal on PBD six, again decreased dramatically by PBD 12 only to show a second recovery towards normal on PBD 24. This same pattern was seen for the subsets of lymphocytes, although T suppressor-cytotoxic cells declined to a greater extent than did Ia+ lymphocytes on PBD 2 and increased to a lesser extent at PBD 6. Note that lymphocyte changes in the peripheral blood were markedly different from those in any other tissue studied.

TABLE 4. *Effect of Time Post-Burn on Percentages of Cells Comprising Lymphocyte Subsets Within Rat Lymphoid Tissues*

Lymphoid Tissue	Sham	Time Post-Burn					
		2 Days	6 Days	12 Days	24 Days	48 Days	60 Days
Spleen							
Ia	42‡	25†	50	43	43	36	44
T	52	44*	45*	54	44*	51	55
$T_{H/I}$	34	28*	29*	32	30	42†	41†
$T_{S/C}$	25	20†	18†	24	17†	18†	22
Thymus							
Ia	5	7	13*	7	14†	20†	15*
T	93	94	85*	92	84†	92	93
$T_{H/I}$	58	72†	48*	53	73†	89†	89†
$T_{S/C}$	94	87	85	91	59†	81	87
Bone marrow							
Ia	16	21	20	11	4†	7†	21
T	16	18	31†	24†	3†	14	16
$T_{H/I}$	5	10†	8†	7†	4	3	12†
$T_{S/C}$	10	10	14	11	3†	4†	11
Cervical nodes							
Ia	42	25†	46	42	27†	42	32
T	52	47	48	54	66†	61*	66†
$T_{H/I}$	36	33	35	34	49†	45†	50†
$T_{S/C}$	20	16	16	21	19	18	25
Mesenteric nodes							
Ia	40	34	39	42	34	44	40
T	61	66	58	59	66	59	64
$T_{H/I}$	48	53	46	47	51	48	52
$T_{S/C}$	22	20	17†	16†	13†	14†	23
Nodes draining burn wound‖							
Ia	26	28	39†	38†	28	37†	38†
T	75	74	62†	61†	72	70	63†
$T_{H/I}$	57	57	45†	44†	55	55	49*
$T_{S/C}$	22	24	22	18†	19*	17†	23
Blood							
Ia	19	29†	25*	13*	22	—	—
T	76	66†	71*	86†	77	—	—
$T_{H/I}$	56	45†	55	68†	58	—	—
$T_{S/C}$	23	22	19†	30†	23	—	—

* $p < 0.05$, † $p < 0.01$ *versus* sham by analysis of variance and Dunnett's test.

‡ Mean per cent immunofluorescence-positive cells.

‖ Nodes draining burn wound: axillary, brachial, inguinal, and lumbar nodes.

Tissue Lymphocyte Subset Percentages (Table 4)

Although Ia antigens are present on activated T cells as well as B cells and monocytes-macrophages, in the absence of activated T cells, the assessment of Ia on scatter-gated lymphocytes should provide an estimate of B-cell percentages. Further, because lymphocytes are comprised almost entirely of T cells and B cells, the sum of the B- and T-cell percentages in a given sample should equal approximately 100. Finally, T helper-inducer cells (CD4+) and T suppressor-cytotoxic cells (CD8+) are mutually exclusive subsets of T cells; therefore, the sum of the percentages of these two cell types should equal approximately the percentage of total T cells (W3/13+) in a given sample. In Table 4, it is demonstrated that these rules held true for all tissues from sham control rats except the thymus and bone marrow. In the thymus, Ia- and T-cell percentages did sum to approximately 100, but the percentages of T helper-inducer cells ($T_{H/I}$) and T suppressor-cytotoxic cells ($T_{S/C}$) summed to much greater than the T-cell percentage. This was not surprising because it has been established that cortical thymocytes coexpress CD4 and CD8 antigens.[7,24] The situation in sham bone marrow was just the reverse of the thymus. The sum of the $T_{H/I}$- and $T_{S/C}$-cell percentages equaled the T-cell percentage, but the sum of the Ia- and T-cell percentages was only 32%, much less than 100%. This indicates that in the bone marrow there are significant numbers of cells with the physical characteristics (light scatter) of lymphocytes that do not express either Ia- or T-cell markers. Whether or

not these cells are lymphocyte precursors or another mature cell type is not known.

In the spleen, percentages of lymphocytes within all four subsets decreased significantly at PBD 2, and the sum of Ia- and T-cell percentages was only 69%, suggesting presence or transport of some unknown cell type with lymphocyte light scatter characteristics into this compartment. Ia+ lymphocytes decreased markedly from a sham level of 45% to 25% on PBD 2. By PBD 6, the Ia+ lymphocyte percentage had normalized, and the sum of the Ia + T percentage also had normalized, reaching 95%. However, percentages of T cells and $T\text{-}_{H/I}$ (CD4+) and $T\text{-}_{S/C}$ (CD8+) cells remained significantly decreased. All subset percentages were normal on PBD 12, a time at which numbers of lymphocytes also had returned to normal (see previous section). On PBD 24, the percentage of splenic T cells declined again, but this decrease was limited only to the $T\text{-}_{S/C}$ subset of T cells. This decline in the $T\text{-}_{S/C}$ subset percentage was maintained on PBD 48, but was accompanied by a significant increase in $T\text{-}_{H/I}$ cells such that the percentage of total T cells was now normal. All subset percentages were in the normal range by PBD 60. It should be noted that at no time after burn did the splenic $T\text{-}_{H/I}$:$T\text{-}_{S/C}$ (CD4:CD8) ratio decline, and in fact, this ratio was increased at several of the time points.

In the thymus on PBD 2 (Table 4), the percentage of cells bearing the CD4 marker increased significantly with no change in percentages of W3/13 or CD8 marker bearing cells. Because, as outlined above, cortical thymocytes coexpress CD4 and CD8 markers and 94% of the cells in the sham group thymus expressed the CD8 marker, the increase in CD4-bearing cells suggests that during the first two days after burn the CD4 marker is induced on CD8+ thymocytes that do not normally express CD4 or there is a change in the thymic environment such that there is selective thymocyte death with remaining cells preferentially expressing the CD4 marker. Further, at PBD 6 the percentage of CD4+ thymocytes decreased significantly relative to sham, and there still was no change in CD8-bearing cells. Thus, during the six days after injury, expression of CD4 on CD8+ cells first increases and then decreases. Percentages of cells in the four subsets were not different from control on PBD 12; note that the same was true for splenic subsets on day 12. Thymic subset percentages changed dramatically on PBD 24 but in a different pattern than during the early postburn period. Ia+ cells were significantly increased, W3/13+ cells decreased, cells bearing the CD4 marker increased as also was seen on PBD 2, and in contrast to earlier time points, CD8+ cells decreased significantly. These last two observations suggest that from PBD 12 to 24 thymocytes coexpressing CD4 and CD8 markers upregulate the CD4 marker while downregulating the CD8 antigen. At PBD 48, the percentage of Ia+ cells increased further to 4-fold over that of control, W3/13+ cells normalized, CD4+ cells remained significantly increased while the expression of the CD8 marker returned to sham levels. At the last time point studied, PBD 60, Ia+ cells were returning towards normal, and the expression of CD4 remained significantly elevated.

As stated above, percentages of Ia+ and W3/13+ cells in the bone marrow did not sum to 100%, indicating that there were significant numbers of bone marrow cells with the light scatter characteristics of lymphocytes that did not express either Ia- or T-cell markers. At PBD 2, the percentage of CD4+ cells increased significantly with no change in any other subsets (Table 4). CD4+ cells remained elevated on PBD six but with a concomitant increase in W3/13-bearing cells, and this pattern persisted at day 12. Relative to controls, a marked decrease in the percentages of cells comprising all subsets except CD4 occurred at PBD 24 such that the sum of Ia + T was only 7% (4% + 3%) *versus* 32% (16% + 16%) for control. On PBD 48, bone marrow subset percentages began to normalize, and by PBD 60 all percentages were normal with the exception of the CD4 subset, which returned to a significant elevation as was observed in the early postburn period.

In the cervical lymph nodes, percentages of Ia+ lymphocytes, presumably B cells, decreased by 40% on PBD 2 but returned to normal by PBD 6. No other significant changes in any other subset percentages were observed until PBD 24. At day 24, percentages of Ia+ cells again decreased by approximately 40% and T cells increased significantly. Further, this increase in T cells could be accounted for entirely by a concomitant increase in CD4+, $T\text{-}_{H/I}$ cells because CD8+, $T\text{-}_{S/C}$ cells remained unchanged. At PBD 48, Ia+ lymphocytes again normalized while percentages of T cells and the CD4 subset of T cells remained elevated. This pattern persisted on PBD 60.

The percentages of lymphocytes comprising the subsets in the mesenteric lymph nodes remained remarkably unchanged throughout the 60-day study period. The one exception to this was the significant decrease in CD8+, $T\text{-}_{S/C}$ cells during PBDs 6 to 48.

In the NDB, there were no changes in subset percentages at PBD 2 (Table 4). However at PBD 6, Ia+ lymphocytes increased significantly while T cells and the CD4 subset of T cells decreased significantly. The same pattern was observed at PBD 12 with the addition of a decrease in the CD8 subset percentage. By day 24, all subset percentages had returned to normal with the exception of CD8, which remained decreased. On PBD 48, NDB Ia+ lymphocytes again increased significantly and CD8 remained depressed. The PBD 60 pattern was similar to that of PBD 6, i.e., increased Ia+ lymphocytes, decreased T and CD4+ cells, and normal CD8+ lymphocytes.

TABLE 5. *Cell Surface Phenotype of Small, Resting Lymphocytes and Large, Blast-like Lymphocytes Obtained from the Nodes Draining the Burn Wound (NDB) at 12 Days Following Thermal Injury*

Marker Expressed	Lymphocyte Type*	
	Small, Resting	Large, Blast-like
Ia	38 ± 5†	84 ± 3
T (sialoglycoprotein)	60 ± 6	83 ± 3
$T_{H/I}$ (CD4)	41 ± 4	34 ± 4
$T_{S/C}$ (CD8)	20 ± 2	13 ± 2
Transferrin Receptor	7 ± 1	51 ± 10
IL-2 Receptor (CD25)	5 ± 1	11 ± 1

* Small, resting lymphocytes were distinguished from large, blast-like lymphocytes on the basis of flow cytometric light scatter characteristics. Mutually exclusive scatter gates were employed to assess each cell type.

† Mean ± SEM per cent immunofluorescence-positive cells per total number counted in each cell population (N = 5/group).

Finally, peripheral blood Ia+ lymphocytes increased at PBD 2 (Table 4). Note that this was in contrast to the spleen and cervical nodes in which Ia+ cells decreased significantly at PBD 2. Also, peripheral blood T cells and CD4+ T cells declined significantly at PBD 2, with CD8+ cells showing no change. At PBD 6, Ia+ lymphocytes remained elevated, and T cells still were decreased relative to control. However, CD4+ T cells normalized with CD8+ T cells now responsible for the decreased percentage of total T cells. By PBD 12, subset changes were the reverse of those observed at PBD 2. Thus relative to sham, Ia+ lymphocytes were decreased significantly and T cells were increased significantly. Both the CD4 and CD8 subsets contributed to the increase in the total T-cell percentage at PBD 12. All subsets had normalized by PBD 24, the last study time for peripheral blood lymphocytes.

Characterization of Large Lymphocytes in the NDB

Numerical and phenotypic lymphocyte changes in the NDB, as well other tissues refer to small resting lymphocytes. But in the NDB, large numbers of cells with the light scatter characteristics of lymphoblasts (forward angle light scatter intensity higher than resting lymphocytes and identical right angle-light scatter intensity to resting lymphocytes) also were noted at PBD 12. In fact, a mean of 40×10^6 of these large cells were present in the PBD 12 NDB, compared to 106×10^6 small lymphocytes (Table 2). This observation prompted further study of these large cells. Unstimulated ^{3}H-thymidine uptake of PBD 12 NDB cells was increased 20-fold over that of cells from sham-injured rats, suggesting that proliferation was occurring in the NDB. However, the absolute magnitude of proliferation was not overly impressive (3210 cpm for burn *vs.* 166 cpm for sham). Phenotypic characterization of the large cells in the 12-day NDB was performed on five rats (Table 5). Because it was hypothesized that these cells were lymphoblasts, expression of two additional markers that should be present on activated lymphocytes were evaluated in this experiment. These markers were assessed with monoclonal antibodies OX-26 (labels the transferrin receptor) and OX-39 (labels the interleukin-2 receptor, CD25 antigen). Scatter gates were set first on small resting lymphocytes and the phenotypic profile was determined. Then the gates were moved to encompass the large cell cluster, and the phenotypic profile of these cells was evaluated (Table 5). The small lymphocyte cluster appeared normal in phenotype. The sum of Ia+ lymphocytes and T cells (38% + 60%) was 98%. Further, the sum of CD4+ and CD8+ cells (41% + 20%) was virtually identical to that of the total T-cell percentage (60%), and the CD4:CD8 ratio was approximately two. Expression of transferrin and Il-2 receptors was 7% and 5%, respectively, appropriate numbers for resting lymphocytes. The phenotype of the large cells was strikingly different from the small lymphocytes. Coexpression of Ia- and T-cell markers was evident with 84% of the cells OX-6+ and 83% positive for W3/13. At first it was thought that these results indicated the presence of T lymphoblasts among the large cells because activated T cells express Ia. However, only 11% of the cells expressed the IL-2 receptor, thus casting doubt on this conclusion. CD4 is expressed on macrophages, and macrophages express Ia to a variable extent as well. Only 34% of the large cells expressed CD4, and the CD4:CD8 ratio was approximately the same as found for the small, resting lymphocytes (macrophages do not express CD8). The light scatter characteristics of the large cells was distinct from macrophages in that the large cells showed less right-angle light scatter intensity than macrophages normally do. Thus, the majority of large cells in the NDB at PBD 12 did not appear to be either T lymphoblasts or macrophages. Although activated B cells, like T cells, now are known to express IL-2 receptors,[25] peripheral B cells do not express the W3/13 marker.[26] Finally, granulocytes express W3/13, but not Ia, and the large cells present in the NDB at PBD 12 definitely did not have light scatter characteristics of granulocytes. Thus, at this time, it is not possible to catagorize these large cells within a definite leukocyte lineage.

Discussion

The data presented demonstrate that following major thermal injury in the rat, strikingly different numerical and phenotypic lymphocyte changes occur in the seven lymphoid compartments evaluated here. In fact, the responses of the seven compartments were so different that it is somewhat difficult to perceive a common pattern of change among them. However, the data do show that all compartments manifested a decrease in TLN at some point during the first six days after burn. From PBD 6 until PBD 60, the spleen, cervical nodes, and NDB then

showed a fairly monotonic increase in TLN with the NDB manifesting the most dramatic increase (up to 8-fold over sham) in TLN. In contrast, lymphopoetic tissues, the thymus and bone marrow, remained severely depleted of lymphocytes until PBDs 60 and 48, respectively, at which times repletion of these tissues ensued. The mesenteric lymph nodes, also reportedly a site of lymphopoesis, manifested a somewhat similar pattern as the thymus and bone marrow, albeit with much less severe depletion of lymphocytes. Finally, the TLN changes in the blood were characterized by abrupt decrease at PBDs 2 and 12 with apparent recovery at PBDs 6 and 24.

Changes in lymphocyte subset percentages in the various compartments can be divided grossly into two phases. In Table 4, it can be seen that all compartments showed significant phenotypic changes in the first six days after burn. With the exception of the NDB and the blood, this was followed by a return towards normal on PBD 12. The second phase then ensued with significant phenotypic changes again occuring in most tissues from PBDs 24 to 60. However, it is thought that care must be exercised in evaluating these changes in subset percentages. In most cases, the variability among animals in subset percentages was very small, leading to highly significant ($p < 0.01$) differences when the change from sham to burn was as small as 2%, for example CD4+ cells in the bone marrow at PBD 12. Although there is no question that such changes were statistically significant, one wonders whether a 2% change in a subset percentage is clinically relevant. This is not to say that all changes in subset percentages were of such a small magnitude; for example the decrease in Ia+ lymphocytes in the spleen and cervical nodes at PBD 2, or the increase in Ia+ cells and decrease in CD4+ lymphocytes in the NDB at PBD 6 (Table 4). Surely changes of these magnitudes in subset percentages would be expected to have immunoregulatory consequences in the tissues in which they occur.

Mouse splenic lymphocyte subset changes following 20% TBSA burn have been reported by Hansbrough et al.[27] At PBD 7 it was found that percentages of T cells (Lyt-1, CD5), $T\text{-}_{H/I}$ cells (L3T4, CD4) and $T\text{-}_{S/C}$ cells (Lyt-2, CD8) decreased significantly, but B cells (sIg+) showed no change. This is the same pattern observed for rat splenic lymphocytes at PBD 6 in the present study (Table 4), although subset percentages apparently differ in magnitude in the mouse as compared to the rat spleen. However, it should be noted that rat splenic B cells did decrease significantly at PBD 2 in the present study, a time point not assessed in the mouse study. Hansbrough et al.[27] also reported that at PBD 15 percentages of T cells, $T\text{-}_{H/I}$ cells and $T\text{-}_{S/C}$ cells remained significantly decreased in mouse spleens. This is in contrast to the present data that showed normal subset percentages in rat spleens at PBD 12 (Table 4).

Burleson et al.[28] investigated rat peripheral blood lymphocyte subpopulation changes two days after 30% TBSA burn injury or burn injury plus exogenous infection with *Pseudomonas aeruginosa* bacteria. For scatter-gated lymphocytes, control values for subset percentages were very similar to those reported here, 72%[28] *versus* 76% in our study for T cells, 58% *versus* 56% for $T\text{-}_{H/I}$ cells, and 21% *versus* 23% for $T\text{-}_{S/C}$ cells (values for the Burleson study estimated from graphic data). However, Burleson et al.[28] found no significant changes in subset percentages at PBD 2 for uninfected rats, while significant decreases of approximately 10% were observed for T cells and $T\text{-}_{H/I}$ cells in the present study (Table 4). It should be noted that a significant decrease in $T\text{-}_{H/I}$ cells was reported for the burned, infected rats in the previous study. It is gratifying that the control subset percentages were virtually identical between the two studies, and the discrepencies could be due to the fact that a 30% TBSA burn was employed in the previous study while a 50% TBSA burn was used in this study. It is possible that the significant decrease in $T\text{-}_{H/I}$ cells in the burned, infected rats in the previous study and the 50% TBSA burned rats in this study was due to the added burden of bacterial infection on one hand and that of the additional 20% TBSA burn on the other hand.

In a more recent report, Burleson et al.[29] extended the above studies to evaluation of lymphocyte subset changes in the spleen and mesenteric lymph nodes of burned and burned, infected rats. Two days after 30% TBSA injury, no changes were observed in percentages of splenic T cells or $T\text{-}_{H/I}$ or $T\text{-}_{S/C}$ subsets. This contrasts with the present report where significant decreases were found in splenic T cells and the $T\text{-}_{H/I}$ subset and a small but significant increase in the percentage of $T\text{-}_{S/C}$ cells. As discussed above, this discrepency may be due to the larger burn size in the present study. Alternatively, splenocytes in the Burleson et al.[29] study were isolated on Ficoll-Hypaque, while in the present study gradient enrichment was not employed. For the mesenteric nodes at two days after burn, Burleson et al.[29] found a significant change, a decrease, only in the percentage of $T\text{-}_{S/C}$ cells, while the current results showed no change in any lymphocyte subsets at this time after injury.

The depletion of lymphocytes at PBD 2 from all lymphoid compartments except the bone marrow deserves comment because this is similar to what is observed with corticosteroid administration in rodents.[30–32] Data from this laboratory[33] have documented extremely elevated levels of corticosterone for the first 48 hours after burn in rats. Thus, the observation of selective depletion of lymphocytes from all tissues except the bone marrow at PBD2 may be mediated by corticosteroids. The mechanisms of these changes has not been elucidated but may involve a decrease in the normal events governing lymphocyte entrance into lymph nodes and splenic tissue *via*

high endothelial postcapillary venules with an increased transit of lymphocytes across the sinusoidal enothelium of the bone marrow leading to sequestration of lymphocytes in this compartment.[32] However, the increase of lymphocytes in the bone marrow at PBD 2 does not account quantitatively for the lymphocyte loss from the other compartments. Total lymphocyte number/rat were calculated from Table 3, multiplying the bone marrow number by 8.5 (about 12% of the bone marrow is contained in two femurs[34]) and the blood lymphocyte count per ml by 20 (approximate blood volume of 350 g rat) and yielded a mean lymphocyte number of 375×10^6/rat for sham-injured controls *versus* 187×10^6/rat for rat at PBD 2. Thus, 50% of lymphocytes cannot be accounted for at PBD 2, and the increase in the bone marrow only accounts for 20×10^6 lymphocytes. This discrepency could be secondary to lymphocyte destruction in the steroid-sensitive rat or redistribution to an as-yet undetermined compartment.

Even if elevation of corticosteroids is related to the lymphocyte changes observed in the two days after injury, other factors must be responsible for subsequent changes because corticosterone levels return to normal by PBD 6.[33] The appearance of increased numbers of lymphocytes in the NDB that begins at PBD 6 and persists until PBD 60 might be related to colonization of the burn wound by microorganisms and/or the inflammatory response. Under normal conditions there is a balanced flux of lymphocytes entering peripheral nodes *via* postcapillary high endothelial venules with those lymphocytes returning to the blood *via* the efferent lymphatics. This normal state of affairs is altered considerably with antigen administration,[3] a situation that may be present in the burned rat with microorganisms colonizing the burn wound. Elegant studies by Hall and Morris[4] have defined the role of lymph nodes in the development and dissemination of the immune response after local antigenic challenge. Localized antigenic stimulation results in sequestration of lymphocytes within the draining lymph nodes.[3–6] This sequestration of lymphocytes has been shown to be a clear consequence of changes in cell-traffic dynamics, i.e., increased entrance and decreased exit rather than cell replication. This phenomenon has been termed "lymphocyte trapping" by Zatz and Lance.[5] Further, the removal of efferent lymph flow from lymph nodes draining the site of antigenic challenge prevents the systemic deployment of the humoral immune response and its development elsewhere in the body. Whether the large increase in TLN in the NDB from PBDs 6 to 60 represents trapping of recirculating lymphocytes or is a result of proliferation of preexisting lymphocytes has not been determined, but based on the above discussion, "trapping" would appear to be a possibility.

However, there is some evidence that proliferation also may account, in part, for the accumulation of lymphocytes in the NDB. This is based on the increased unstimulated ^{3}H-thymidine uptake of lymphocytes from PBD 12 NDB as compared to sham-control cells from the same nodes. In addition, the NDB contained significant numbers of large cells that may be lymphoblasts, although definitive characterization still is lacking. Whatever the case, it does appear that proliferation and/or differentiation of lymphocytes, either T or B, is occuring in the NDB at PBD 12.

Concomitant with the progressive increase in NDB lymphocyte numbers is a persistent decline in lymphocyte numbers in the thymus and bone marrow. The thymic contribution to peripheral lymphoid mass is controversial but has been estimated normally to be 1% per day.[35] It is probable that this thymic export changes dramatically under conditions of adversity such as severe injury because stress, in general, is associated with thymic involution.[36–38] Osmond[39] has shown that, under normal circumstances, small lymphocytes leaving the bone marrow circulate briefly and then home preferentially to the spleen where they reside from one to three days and undergo further maturation before appearing in the peripheral lymphoid organs. However, if trapping of lymphocytes occurs in the NDB, the call for lymphocytes from the bone marrow to the periphery may be increased such that depletion of marrow lymphocytes occurs. This situation would be analogous to that of depletion of bone marrow granulocyte storage pools during peripheral granulocytosis.

Others[40] have described a correlation between peripheral blood lymphocyte count and septic events and death. Although little immunologic "work" actually is performed in the peripheral blood and the status of this compartment may not reflect the condition of the immune system as a whole, TLN in a given lymphoid compartment indeed may reflect in part the immunologic capacity of the compartment and thus its ability to eliminate invading microorganisms or antigen. This idea is supported by the above-cited studies demonstrating sequestration of lymphocytes in lymph nodes draining the site of antigen administration.[3–6] Antigen-induced sequestration of lymphocytes might be especially applicable to regional lymph nodes that drain the burn wound following thermal injury, as is suggested by by the present data in which TLN in the NDB increased up to 8-fold over sham-injured animals. If TLNs in these nodes increase sufficiently due to antigenic stimulation from the burn wound (microorganisms, heat-denatured proteins, and so on) the ability to respond and to contain antigen may be enhanced.

In summary, the changes in the distribution, number, and phenotype of lymphocytes among the various lym-

phoid compartments studied at various times after burn in an uninfected animal model demonstrate the normal migration and/or proliferation of lymphocytes toward the generation of an immune response. Changes within the initial 48 hours after injury may be secondary to glucocorticoid elevation. Thereafter, lymphocytes accumulate in the regional lymph nodes that drain the burn wound. Efforts directed at modulation of the immune response should take into account these profound regional changes in the immune system.

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