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The Effect of Thermal Trauma in Mice on Cytotoxicity of Lymphocytes (39607)

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Burn trauma produces an impairment of cellular immunity manifested by a prolongation of skin graft survival time, by a decrease in the delayed hypersensitivity response, and by a diminished graft versus host reaction (1-3). Since lymphocytes are important mediators of the cellular immune response, a study of their functions could elucidate the mechanisms of this phenomenon. Previous investigations on the effect of thermal trauma on several of the many functions of lymphocytes have shown a defect in the proliferative response of lymphocytes in the mixed lymphocyte culture reaction (4, 5) but no defect in their response to mitogenic stimulation by phytohemagglutinin (6, 7).

In this communication, we studied the effect of thermal injury on the cytotoxicity of lymphocytes, a property which correlates with the survival time of skin allografts.

Materials and methods. Inbred, adult, female BALB/c mice (h-2^d) and male C57BL/6N mice (H-2^b) were used as host and donor, respectively. The murine ascites tumor, EL-4, carried in adult C57BL/6N mice by passage once a week, was used as immunizing material and target cells. Mice were sensitized by a single ip injection of 3×10^7 tumor cells in 0.1 ml of phosphate-buffered balanced salt solution (BSS). Lymphoid cells from spleen and mesenteric lymph nodes were harvested as previously described (8). Spleens from two or three mice and lymph nodes from four mice were pooled for each experiment. For each assay of lymphoid cells from peripheral blood, 10 identically treated BALB/c mice were decapitated and bled into 0.5 ml of heparin. The blood was diluted with an equal volume of BSS, and the lymphocytes were separated by a Ficoll-Hypaque gradient.

For the cytotoxic assay, previously described methods (8, 9) with some modifications were used. One milliliter of attacking cells (at concentrations of 4, 2, 1, or 0.5×10^6 cells/ml of Eagle's minimal essential medium with 10% heat-inactivated fetal calf serum) was pipetted into plastic tissue culture tubes (No. 3033 Falcon Plastics) at 4°. Afterwards 1 ml of ^{51}Cr-labeled target cells (5×10^5 cells/ml of medium) was added to each tube. All analyses were performed in quadruplicate. The tubes were centrifuged at 65*g* for 5 min at room temperature in an International centrifuge (Model V, Size 2). The tubes were then incubated at 37° in the presence of 5% CO_2 in air for 4 hr. Next the tubes were centrifuged at 1500*g* for 15 min at 4° in an International refrigerated centrifuge (Model PR-2). A 1-ml aliquot of the supernatant fluid was pipetted into a small glass tube and covered with parafilm. Radioactivity was measured in a Packard gamma scintillation spectrometer (Model 578).

The results are expressed as the percentage of maximal ^{51}Cr release (% lysis) determined by freezing and thawing ^{51}Cr-labeled target cells four times. Average control ^{51}Cr release was measured by incubating target cells in the presence of nonsensitized lymphocytes from BALB/c mice. To calculate the percentage lysis:

$$\%\ \text{Lysis} = \frac{\text{Average test } ^{51}\text{Cr release} - \text{Average control } ^{51}\text{Cr release}}{\text{Freeze-thaw } ^{51}\text{Cr release} - \text{Machine background}} \times 100.$$

Comparison between the cytotoxic ability of lymphocytes obtained from sensitized unburned and burned mice was made using the following ratio:

$$\frac{\%\ ^{51}\text{Cr release due to } 2 \times 10^6 \text{ attacking cells/ml from burned mice}}{\%\ ^{51}\text{Cr release due to } 2 \times 10^6 \text{ attacking cells/ml from unburned mice}}$$

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Anesthetized BALB/c mice were given a two-thirds body surface area burn in water at 70° for 7 sec and were treated with 3 ml of 0.85% NaCl sc. The anesthesia produced no statistically significant effect on cytotoxicity of lymphocytes.

In the preincubation experiments (Table I), 0.4 ml phosphate-buffered saline (PBS), pH 7.4,0.4 ml of mouse sera, or 0.1 ml of hydrocortisone (HC: hydrocortisone sodium phosphate, Merck, Sharp & Dohme) were added to each tube of 1 ml of attacking cells obtained from the spleens of normal mice 10 days after sensitization (Table I, column A) and allowed to stand for 1/2 hr at room temperature before adding 1 ml of target cells. The cytotoxic assay was then carried out. In Table I, column B, the same quantities of PBS, serum, or HC were added to each tube of attacking cells and incubated at 37° for 1 hr with shaking. Afterwards, the cells were washed two times with 1 ml of Eagle's medium. Then the attacking cells were reconstituted with 1 ml of Eagle's medium before addition of 1 ml of target cells, followed by the cytotoxic assay. In Table I, column C, the same procedure was used with 1 ml of target cells as described for attacking cells in B, but in the last step, 1 ml of attacking cells was added after reconstitution of target cells.

For statistical analysis, the probability of 0.05 ($P = 0.05$) was chosen as the level of statistical significance. Statistical differences between groups were calculated with the Student's t test. The data in Figs. 2 and 3 were plotted as a ratio of burned:normal for the sake of clarity and brevity, and statistical significance for the difference between the means of the quadruplicate samples of burned and unburned mice was calculated for each point of the figure.

Results. The effect of thermal trauma on cytotoxicity of sensitized lymphocytes. Preliminary experiments on normal BALB/c mice sensitized with EL-4 tumor cells showed a linear relationship between ^{51}Cr release from target cells and attacking cells at a concentration of 5×10^5 cells per tube for target cells and at a concentration of 2, 1, and 0.5×10^6 cells per tube for spleen and peripheral blood lymphocytes, or at 4, 2, and 1×10^6 cells per tube for mesenteric lymph-node lymphocytes. Figure 1 illustrates the typical development of cytotoxic cellular immunity with time in unburned

TABLE I. EFFECT OF POSTBURN SERUM AND HYDROCORTISONE ON CYTOTOXICITY OF SPLEEN CELLS FROM UNBURNED, SENSITIZED MICE.

Additions	A No preincubation at 37° (% lysis) Mean ± SD	B Preincubation of attackers at 37° (% lysis) Mean ± SD	C Preincubation of targets at 37° (% lysis) Mean ± SD
PBS	29.7 ± 0.56	23.9 ± 1.88	23.4 ± 1.59
Normal mouse serum	36.9[a] ± 1.81	36.4[a] ± 1.15	25.2 ± 1.40
1-Hr postburn serum	34.5[b] ± 1.91	30.3[a] ± 1.27	26.4 ± 2.35
2-Hr postburn serum	31.3 ± 3.48	23.0 ± 0.50	
PBS	49.1 ± 0.61	36.6 ± 1.63	33.9 ± 1.88
Normal mouse serum	57.2[a] ± 0.49	45.0[a] ± 1.78	35.5 ± 0.70
1-Day postburn serum		41.6[b] ± 1.50	31.8 ± 0.92
4-Day postburn serum		41.7 ± 3.04	34.0 ± 2.90
PBS	55.4 ± 3.1	45.1 ± 0.7	50.8 ± 1.7
HC 480 μg/ml	50.5[c] ± 2.0	37.8[a] ± 1.9	49.7 ± 2.4
HC 48 μg/ml	55.2 ± 1.6	41.1[a] ± 1.9	47.7 ± 1.8
HC 4.8 μg/ml	52.9 ± 2.3		
HC 0.5 μg/ml	57.5 ± 0.5		
HC 0.05 μg/ml	58.0 ± 1.3	40.6[b] ± 2.3	49.1 ± 1.6

[a] $P < 0.01$.
[b] $P < 0.02$.
[c] $P < 0.05$.
Statistical significance was calculated on the difference between each value in the column and its corresponding control with PBS.

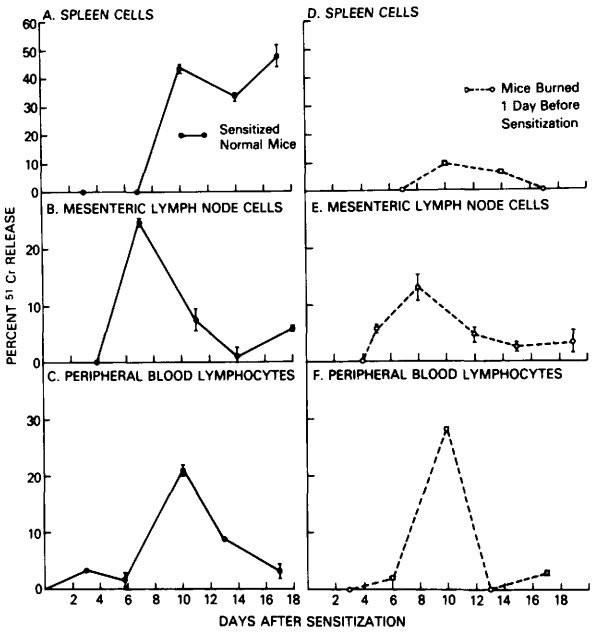

FIG. 1. The development of cytotoxic lymphocytes of BALB/c mice with time after sensitization with EL-4 tumor cells. The cytotoxicity assay was carried out with 2×10^6 attacking cells from the spleen and peripheral blood, but with 4×10^6 attacking cells from mesenteric lymph nodes. All tubes contained 5×10^5 target cells. All analyses of each organ were performed on the same day, but the comparison between organs or between normal and burned mice was not simultaneous. The burn is described in Materials and Methods. Each point represents the mean ± SD of pooled organs, as described in Materials and Methods, for one experiment. The same kinetics were obtained when the experiment was repeated two to three times.

and burned mice. In unburned mice, there was a sharp increase in cytoxicity around 10 days after sensitization in the case of spleen and peripheral blood lymphocytes (A and C), whereas maximum cytotoxicity of mesenteric lymph node lymphocytes from these mice occurred slightly earlier, around 7 days after sensitization (B). When BALB/c mice were burned 1 day before sensitization, there was no essential change in the time required for development of peak cytotoxic activity (Fig. 1D-F). For this reason, *in vitro* studies were usually performed with lymphocytes collected at 7 or 10 days after sensitization, depending upon the source.

Figure 2 shows the effect of thermal trauma given at various times before or after sensitization on the cytotoxic cellular immune response. In Fig. 2A, the results demonstrate a significant decrease of cytotoxicity when the burn was given any time from 7 days before sensitization to 13 days after sensitization ($P < 0.05$ with all ratios < 0.8). In a few experiments, however, there was evidence of enhancement at the extremes of the time scale studied, i.e., when the burn was given at 14 days before sensitization or at 12 or 13 days after sensitization ($P < 0.05$).

In Fig. 2B, a significant decrease ($P < 0.05$) of cytotoxicity of mesenteric lymph node cells was observed when the burn was given from 1-7 days after sensitization. Contrary to the findings with spleen cells, thermal trauma before sensitization or at 8 days or later after sensitization did not reduce cytotoxicity in most cases. Significant enhancement occurred in only two ratios above 1.4 ($P < 0.01$).

In Fig. 2C, a significant fall ($P < 0.01$) of cytotoxicity of peripheral blood lymphocytes was found when thermal injury was applied from 4 days before sensitization to 6 days after sensitization. Here again, in contrast to the findings with spleen cells, burning 8-10 days after sensitization did not re-

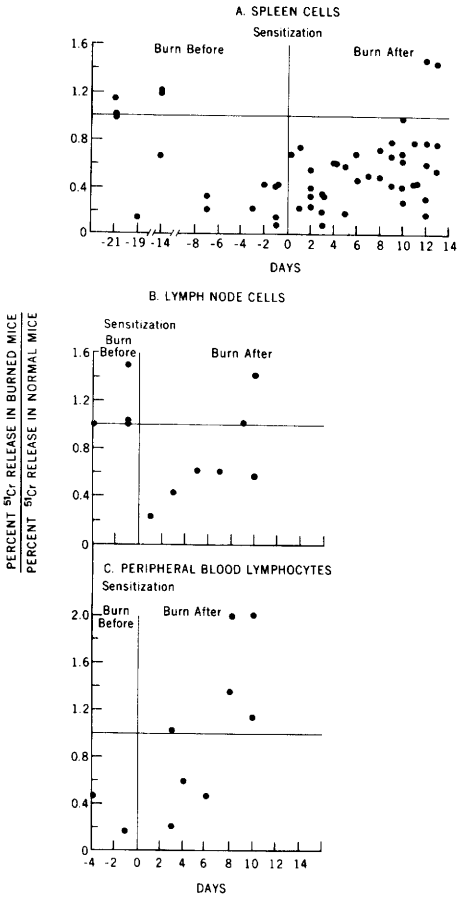

FIG. 2. The effect of thermal trauma on cytotoxicity of 2 × 10^6 lymphocytes of BALB/c mice sensitized with EL-4 tumor cells. Each point refers to a separate experiment comparing pooled samples (see Materials and Methods) from burned and unburned mice. The burn is described in Materials and Methods. Spleen cells were harvested in normal and burned mice 10–11 days after sensitization, except when the burn was given after 9 days. In the latter case, harvesting was delayed in both burned and unburned mice until 1–3 days after thermal trauma.

duce cytotoxicity of peripheral blood lymphocytes. Significant enhancement occurred in only two experiments with ratios around 2 at 8 and 10 days ($P < 0.01$).

The effect of burn serum on cytotoxicity of sensitized lymphocytes from normal mice. Table I shows a typical experiment of the effect of postburn sera, as well as normal mouse sera, on the cytotoxicity of spleen cells collected from normal, sensitized mice. In column A, both normal and 1-hr postburn sera showed enhancement of cytotoxicity ($P < 0.05$). Enhancement also occurred when these sera, in addition to 1-day postburn serum, were preincubated with attacking cells (column B). In both cases, the enhancement was less with 1-hr and 1-day postburn sera than with normal sera. Neither normal nor postburn sera showed any significant effect on preincubation of target cells with sera before the assay (column C).

The effect of hydrocortisone on cytotoxicity of sensitized lymphocytes from normal mice. Table I shows that hydrocortisone was able to reduce cytotoxicity by 9% at a concentration of 480 μg/ml when there was no preincubation at 37° ($P < 0.05$). When hydrocortisone was preincubated with attacking cells, cytotoxicity was decreased by 16% at a concentration of 480 μg/ml, but even by 10% at a concentration of only 0.05 μg/ml ($P < 0.02$). The drug had no significant effect (2–6% inhibition) on preincubation with target cells.

The effect of lymphocytes from burned animals on cytotoxicity of sensitized lymphocytes from normal mice. Table II shows the results of experiments designed to detect "suppressor" cells. When lymphocytes from the spleens of burned mice were added to the cytotoxicity assay, there was a significant inhibition of cytotoxicity by spleen cells collected on Days 1, 2, 3, 9, 12, and 16 after burning (column A compared with column C); however, the addition of spleen cells from normal mice to the assay also showed the same phenomenon (column B compared with column C). Only on 3- and 9-days postburn was the difference between cells from burned and normal mice significantly different ($P < 0.05$) (column A compared with column B). These results indicate that burn injury does not produce a significant number of "suppressor" cells in the spleen which affect function of the preformed cytotoxic cells.

Discussion. The decreased cytotoxicity of spleen and lymph node cells, as well as peripheral blood lymphocytes from burned animals, could be responsible for the prolonged survival time of skin allografts after thermal injury. The effects of burning are

TABLE II. EFFECT OF NONSENSITIZED SPLEEN CELLS FROM BURNED AND NORMAL MICE ON CYTOTOXICITY OF NORMAL, SENSITIZED SPLEEN CELLS.[a]

Days postburn when spleen cells were harvested	A Normal sensitized + burn (% lysis)	B Normal sensitized + normal (% lysis)	C Normal sensitized alone (% lysis)
	Mean ± SD	Mean ± SD	Mean ± SD
1	15.3[c] ± 0.76	14.6[c] ± 0.99	18.2 ± 0.33
2	27.0[d] ± 1.06	27.3 ± 1.39	29.3 ± 0.82
3	30.2[b] ± 1.62	33.3[b] ± 0.94	38.9 ± 0.65
4	9.0 ± 1.94	8.6[e] ± 0.35	10.2 ± 0.70
7	9.1 ± 1.01	8.6[e] ± 0.35	10.2 ± 0.70
9	12.8[b] ± 0.17	14.6[c] ± 0.99	18.2 ± 0.33
12	33.2[b] ± 0.48	33.3[b] ± 0.94	38.9 ± 0.65
16	26.0[e] ± 1.73	27.3 ± 1.39	29.3 ± 0.82

[a] Cytotoxicity was measured using 2×10^6 spleen cells from mice sensitized with EL-4 tumor cells and 5×10^5 ^{51}Cr-labeled tumor cells. Additions listed were: burn, 2×10^6 spleen cells from burned nonsensitized mice; normal, 2×10^6 spleen cells from normal nonsensitized mice. Nonsensitized spleen cells from normal or burned mice produced an 8–11% release of ^{51}Cr in the cytotoxicity assay with 5×10^5 labeled target cells.

[b] $P < 0.001$.

[c] $P < 0.01$.

[d] $P < 0.02$.

[e] $P < 0.05$.

For the calculation of statistical significance, the mean values of columns A and B were compared with the control values of column C.

immediate and potent, at least in the case of spleen cells, since burning at 10–12 days after sensitization, when cytotoxicity has reached a peak, could significantly decrease that cytotoxicity. This was not usually the case with lymph node cells or peripheral blood lymphocytes.

An attempt was made to identify humoral factors in the blood of burned mice which could reduce cytotoxicity of lymphocytes, but no such evidence could be found either in serum or in plasma obtained from several hours to 4-days postburn. Because hormones, especially from the adrenal cortex, can affect cellular immunity (10), and because increased adrenal activity after trauma has been extensively documented (11), hydrocortisone, one important product of that activity, was tested on cytotoxicity of spleen cells. The drug inhibited cytotoxicity of lymphocytes at a concentration as low as 0.05 μg/ml when attacking cells were preincubated with the drug, a level of corticosteroid that occurs in the blood of normal and burned rats and humans (10, 12); however, since the inhibition was only 10%, it is doubtful whether cortisone plays an important part in the much greater inhibition observed in the *in vivo* experiments.

Since efforts to explain the marked reduction of cytotoxicity of lymphocytes by humoral factors failed, a search was made for suppressor cells in the spleens of burned mice to account for this phenomenon. Again no evidence was found to support this mechanism. Since it is possible that an inhibitor of cytotoxicity could be bound to the cell membrane of lymphocytes, experiments are underway to test this hypothesis.

Summary. The effect of thermal trauma on cellular immunity was studied in inbred mice using an *in vitro* assay for cytotoxic lymphocytes from spleen, lymph node, and peripheral blood. Burned BALB/c mice sensitized with EL-4 tumor cells from C57BL/6N mice showed a significant decrease in cytotoxicity of spleen, lymph node, and peripheral blood lymphocytes. This depression of cytotoxicity occurred in spleen lymphocytes when the burn was given as early as 7 days before sensitization or as late as 13 days afterwards. Similar results were obtained with peripheral blood lymphocytes, but in the case of lymphocytes from lymph nodes diminished cytotoxicity was observed only when the animals were burned after sensitization. No evidence was found for an inhibitor of cytotoxicity in serum from burned mice or for "suppressor" cells in the spleen after thermal injury. The

reduced cytotoxicity of lymphocytes in burned animals could be of critical importance in the impairment of some manifestations of cellular immunity after burn trauma.

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