PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 160, 468–472 (1979)

Effect of Burn Trauma in Mice on the Generation of Cytotoxic Lymphocytes (40472)

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Burn injury produces an impairment of cellular immunity (1–4). Since T cells are important mediators of cellular immunity, *in vitro* tests for T cell function are being used to elucidate the mechanism of this phenomenon. Two common *in vitro* assays for cellular immunity measure cytotoxicity or the proliferative response to mitogens and allogeneic cells. In the present experiments, we have used the cytotoxic assay. Previously we found decreased cytotoxic activity of sensitized lymphocytes from spleen, abdominal lymph nodes, and peripheral blood in burned mice (5). In the latter studies we found evidence for a defect of the effector side of the immune response, since sensitized animals burned at the peak of their cytotoxic response showed a significant decrease of cytotoxic activity of spleen lymphocytes. An immuno-suppressant was suspected as the cause of this acute change in cytotoxicity, but no evidence for a serum factor or suppressor cell could be demonstrated (5).

For that reason we have turned our attention to possible defects on the afferent side of the immune response. The mixed lymphocyte culture (MLC) measures this aspect of immunity *in vitro*. Previous investigators studying burned patients reported a defect of both responding and stimulating capabilities of peripheral blood lymphoid cells in the MLC (6, 7). The present experiments study the effect of burning on the sensitization process of lymphocytes from mouse spleen and peripheral blood measured by the cytotoxic assay rather than by the incorporation of radioactive thymidine into DNA used by other authors. Because of the prime role of T cells in this reaction, T cells were separated in some experiments in order to investigate the effect of burning on the cytotoxicity of these cells specifically.

Materials and methods. Animals. Inbred, adult mice, female BALB/c (H-2^d) and male C57BL/10J (H-2^b) were used as host and donor, respectively, in the *in vivo* sensitization experiments.

Sensitization. The murine ascites tumor, EL-4, carried in adult C57BL/10J mice by passage once a week, was used as immunizing material and target cells. Mice were sensitized by a single intraperitoneal injection of 1×10^7 tumor cells in 0.1 ml of Eagle's minimal essential medium.

Thermal trauma. Anesthesized BALB/c mice were given a moderately severe two-third body surface area burn in water at 70° for 7 sec and were treated with 3 ml of 0.85% NaCl subcutaneously. Control mice were anesthetized and given the same amount of saline.

Spleen suspensions. Spleens from two to twenty mice were pooled for each experiment. In the sensitization experiments, the spleen cells were harvested 10–11 days after sensitization.

Cytotoxic assay. As described previously (5). *MLC.* The Mishell and Dutton mixed lymphocyte culture was modified as described by Hodes *et al.* (8). Spleen or T cells from allogeneic mouse strains were used in the MLC. The one-way MLC was carried out by irradiating stimulating cells with 2000 rads. A mixture of 1 ml each of responding cells (2×10^6) and stimulating cells (0.5×10^6) was incubated in the wells of a Linbro tray (Linbro Scientific Inc., New Haven, CO) at 37° with moisturized 5% CO_2 in air for 5–6 days. A control MLC contained 2×10^6 spleen or T cells from burned or nonburned BALB/c mice and 0.5×10^6 irradiated cells from the same inbred strain. The cells were harvested, and viable cells were counted using trypan blue exclusion. Then 1 ml of viable sensitized cells (2×10^6) was added to 1 ml of viable ^{51}Cr labeled target cells (5×10^5), and the cytotoxic assay was performed. Target cells for BALB/c responding cells sensitized against C57BL/10 cells were ^{51}Cr labeled EL-4 cells; for C57BL/10 anti BALB/c responses,

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^{51}Cr labeled BALB/c spleen cells were used as targets. When BALB/c T cells were used as the responding cells in the MLC, irradiated C57BL/10 spleen cells were used as stimulator cells, because allogeneic T cells were poor stimulating cells as determined by cytotoxicity assays after the MLC.

Cell separations. T cells were separated from spleen cells on a nylon wool column as described previously (9). Peripheral blood lymphocytes were separated on a Ficoll-Hypaque gradient (5).

Statistical analysis. The probability of 0.05 ($P = 0.05$) was chosen as the level of statistical significance. Computations of paired data were made for the t value of two groups with the Student's t test. The data in the figures were plotted as the ratio of burned/non-burned, and statistical significance for the difference between the means of the quadruplicate samples of burned and non-burned mice was calculated for each point on the figure. When the term "significant" is used, it refers to statistical significance at the $P = 0.05$ level.

Results. Effect of burn injury on T cell cytotoxicity. Figure 1 demonstrates that splenic T cells collected from animals 1 to 9 days postburn have a significant reduction in cytotoxicity compared with normal controls ($P < 0.05$). The ratios illustrated in Fig. 1 were lower, for the most part, than values obtained from simultaneous experiments performed (data not shown) using cells from unfractionated spleen.

Effect of burn injury on the sensitization of cells in the MLC. Figure 2 shows the cytotoxic

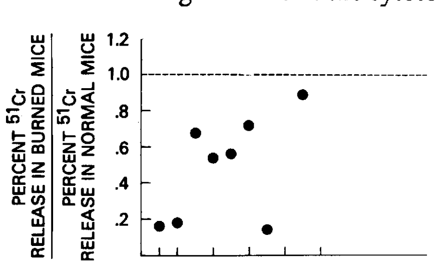

FIG. 1. Effect of burn on spleen T cell cytotoxicity. Each point represents one experiment. A control assay was performed with nonsensitized BALB/c T cells, and the % ^{51}Cr released was subtracted from the values obtained with splenic T cells from burned or nonburned sensitized mice.

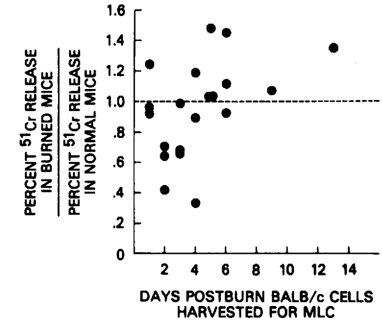

FIG. 2. Effect of burn on generation of cytotoxic splenic T cells in the MLC. Each point represents one experiment. The values used in calculating the ratio were obtained by subtracting the % ^{51}Cr release of the control culture from the experimental culture.

response of T cells obtained from spleens of burned and non-burned mice incubated in the MLC in order to determine the effect of burn trauma on the capacity of T cells to respond to immunogenic stimulation. Cytotoxicity generated by T cells from the spleens of burned mice was significantly lower ($P < 0.05$) than non-burned controls in 100% of the experiments on the second day postburn, in 67% on the third and fourth days postburn, and in 33% on the sixth day postburn. Significant enhancement occurred in 33% of the experiments on the first, fourth, and fifth days postburn, in 67% on the sixth day, and in 100% on the thirteenth day. Poor generation of cytotoxic activity did not correlate with poor viable cell recovery and thus could not be attributed to nonspecific cell death.

No such effect was found, however, with unfractionated cells from spleens of burned mice in the one-way MLC. In the case of responder cell activity from burned mice, only 2 of 18 experiments (11%) demonstrated low cytotoxicity ($P < 0.05$) with cells collected during the 21-day postburn period, while the majority, or 56%, had increased cytotoxicity ($P < 0.05$) compared with normal controls. In the case of stimulator activity of spleen cells from burned mice, only 4 of 17 experiments (24%) with cells collected during the same postburn period showed significant reduction of cytotoxicity. Most responses were within normal limits, while 12% had significantly elevated levels of cytotoxicity ($P < 0.05$).

Peripheral blood cells from burned mice

were also tested for their capacity to respond to immunogenic stimulation in the one-way MLC. In 8 experiments performed with cells collected during the first 12 days after burning, only one experiment with cells collected on the sixth postburn day showed a significantly low cytotoxicity. In 6 of the 7 remaining experiments, the cytotoxic response was increased over that of non-burned mice.

Search for immunosuppressants after burn trauma. To test for circulating factors, 0.5% mouse serum from either non-burned or burned mice was added to the medium in the MLC instead of 10% heat-inactivated fetal calf serum. After 6 days of incubation, the spleen cells were harvested, and the cytotoxic assay was performed. No evidence was found for an immunosuppressant of cytotoxicity in serum collected from burned mice at 1, 2, 18 or 42 hr postburn.

To test for the presence of suppressor cells in burned mice, spleen cells or T cells separated from the spleens of BALB/c burned or nonburned mice were added in concentrations of 0.5, 1 or 2 × 10^6 cells to the MLC containing non-burned BALB/c (2 × 10^6 cells) and irradiated C57BL/10 (0.5 × 10^6) spleen cells. There was no evidence of significant suppression either by T cells or an unfractionated population of spleen cells obtained from burned mice at 2, 3 or 4 days postburn.

To test for the possibility of an immunosuppressant factor adsorbed to the cells, spleen cells from burned mice were incubated from 2 to 6 days in tissue culture medium, after which the cells were harvested and separated by centrifugation. When the supernatant solution was added at various concentrations to the standard MLC with cells from non-burned animals, there was no evidence of suppression of cytotoxicity.

Effect of thermal injury on donor mice cells used for sensitization. Figure 3 shows that burning donor mice can affect the ability of tumor cells from those mice to sensitize recipient mice when measured by spleen cell cytotoxicity. When a burn was given to tumor bearing C57BL/10 mice at any time between transfer of EL-4 cells to C57BL/10 hosts and sensitization of recipient mice, there was a significant decrease ($P < 0.05$) in immunogenicity of the tumor cells in 5 of 7 experiments (71%). In two experiments, there was enhancement ($P < 0.02$). When donor C57BL/10 mice were given a burn 1 day before transfer of EL-4 tumor cells, there was further evidence for suppressed immunogenicity of tumor cells in 2 of 4 experiments, while in one experiment there was enhancement ($P < 0.01$).

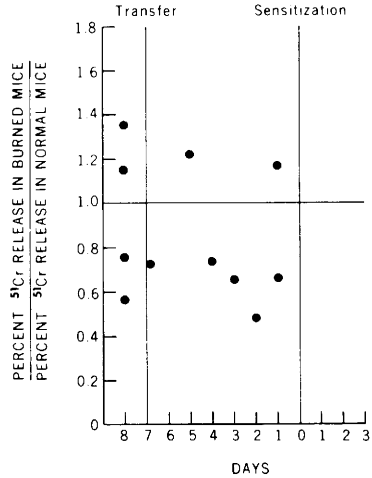

FIG. 3. Effect of burning C57BL donor mice on immunogenicity of EL4 cells injected into recipient BALB/c mice. Days are listed in relation to the time C57BL mice were burned prior to their use for sensitization of BALB/c mice. Each point represents a separate experiment.

To test the effect of heat directly on EL-4 tumor cells, 3 × 10^7 EL-4 tumor cells/ml buffered saline solution were heated in a water bath at 70° for 7 sec and then injected intraperitoneally into BALB/c mice to sensitize them. In this case, however, a 37% increase in cytotoxicity ($P < 0.001$) was noted in recipient spleen cells compared with controls, rather than the decrease noted by burning the donor animal.

Discussion. The results of these experiments indicate that burn trauma alters the sensitization process of lymphocytes on the afferent side of the immune response. Evidence for this conclusion comes from two types of experiments: First, the decreased cytotoxicity generated by T cells from spleens of burned mice incubated in the one-way MLC indicates a defect in the responding capability of T cells to antigenic stimulation as a consequence of burn injury; second, the decreased cytotoxicity observed with spleen cells from mice sensitized with donor cells from burned mice points to a defect of immunogenicity (or stimulating capability) of cells as a result of thermal trauma. These findings partially elucidate the mode of action of immunosuppressive events occurring in mice as a result of burns that cause impairment of cellular immunity (1–4).

The present experiments, however, raise some intriguing questions which cannot be answered definitively at this time. For example, why did the unfractionated population of spleen cells or peripheral blood cells from burned mice not show the reduction of cytotoxicity found with isolated T cells? Perhaps other cells in the unfractionated population counteract or mask the defect in T cells. That this might be the case is suggested by the observation of the lower values of cytotoxicity of effector T cells than with the unfractionated population of spleen cells (see first section of results). Also, can the defect of T cells lasting only a few days explain the *in vivo* findings of a longer period of impaired cellular immunity? Again, perhaps a preparation of T cells without contamination by other cells might demonstrate a defect for a longer period. Another possibility is that burn injury may act at various stages of the immune process rather than at just one, i.e. on effector lymphocytes as well as on the sensitization process. Or, why does heating of donor cells directly increase cytotoxicity whereas donor cells from burned animals show a decreased cytotoxicity? It appears that factors other than heating *per se* are responsible for this phenomenon.

Our results with the cytotoxic assay can be compared with the assay for the proliferative response studied by others. Leguit *et al.* (6) and Sakai *et al.* (7) demonstrated a decreased proliferative response of peripheral blood lymphocytes from burned patients after incubation in the MLC. In those reports there was a defect in both responding and stimulating capabilities. In their experiments an unfractionated population of cells from humans in which the proliferative response was studied gave results similar to ours with an enriched population of T cells in mice in which cytotoxicity was investigated. It appears, therefore, that burn trauma causes impairment in the generation of T lymphocytes involved in both cytotoxic and proliferative functions.

The manner by which burn trauma in mice produces defects in the cytotoxic response of effector lymphocytes (5) and in the generation of cytotoxic lymphocytes described here remains a mystery at this time. All attempts thus far to demonstrate humoral factors in the serum or suppressor cells in the spleens of burned mice have failed. In burned patients, on the other hand, Constantian (10) has reported the isolation of a polypeptide from the serum of burned patients that suppresses the stimulation of peripheral blood lymphocytes by phytohemagglutinin. Whether this polypeptide is produced by the injury or by infecting bacteria is not yet clear. Further efforts in our laboratory will be directed toward the search for humoral factors or suppressor cells by varying: (a) the strains of mice used, (b) the conditions of the assay, and (c) the severity and type of burn studied.

Summary. The mechanisms of the impaired cellular immune response after a moderately severe burn in mice was studied. T cells from spleens of burned BALB/c mice showed decreased cytotoxicity ($P < 0.05$) during the first 9 days postburn compared with nonburned controls. In the one-way MLC, T cells from spleens of the majority of burned BALB/c mice collected on the second, third, and fourth days postburn incubated for 6 days with irradiated spleen cells from C57BL/10J mice also had decreased cytotoxicity ($P < 0.05$). On the other hand, mixed populations of cells obtained from spleen or peripheral blood of burned BALB/c mice demonstrated no defect in sensitization, either in responding or stimulating abilities, in the MLC. Other experiments in which EL4 cells from burned donor C57BL mice were used to sensitize nonburned BALB/c mice showed decreased immunogenicity in recipient mice. These results indicate that the mechanism of the impaired cellular immune response after burn injury, when assayed by cytotoxicity, probably involves defects in the sensitization process, compromising both responding and stimulating capabilities. The changes produced in T cells by burning to cause this phenomenon are not clear, but no evidence was found for humoral factors in the serum of burned animals or for the presence of

suppressor cells which could affect sensitization in the MLC.

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Received September 28, 1978. P.S.E.B.M. 1979, Vol. 160.