Generation of Suppressor Cells in Mice after Surgical Trauma

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A B S T R A C T Immunoincompetency is often seen in patients after various types of trauma and is associated with increased morbidity and mortality from infectious complications. To understand better the immunologic impairment associated with trauma, we have studied this phenomenon in an animal model. Splenocytes from mice traumatized by amputation of their right hind limbs were consistently shown to have a diminished capacity to proliferate in response to alloantigens and to form alloreactive cytolytic cells in mixed lymphocyte cultures. Anesthesia itself had no effect in this system. The immunoincompetency was detected from 2 h to 6 d after surgical trauma and was completely reversed by removing adherent and phagocytic cells from the splenocytes. Furthermore, addition of splenocytes from traumatized mice to mixed lymphocyte cultures from normal mice prevented normal lymphocytes from responding to alloantigens, suggesting the existence of suppressor cells. The suppressor cells were found to adhere to glass and to nylon wool columns, and were contained within an esterase-positive cell population. They were insensitive to treatment with anti-Thy 1.2 and anti-Ig sera in the presence of complement. Therefore, the present results suggest that a Thy 1.2-negative, Ig-negative, esterase-positive cell population capable of adhering to glass and nylon wool, presumably macrophages, was responsible for the inhibition of the responsiveness of lymphocytes to alloantigens in traumatized animals.

INTRODUCTION

Trauma is often accompanied by a loss of immunocompetency, which may contribute to morbidity and mortality from infectious complications. Shortly after major surgical operations, accidental trauma or burns, patients are found to have impaired cell-mediated immunity including anergy to skin test antigens (1, 2). The phagocytic capability of macrophages (3) and neutrophils (4) is diminished. Some investigators also report decreased levels of immunoglobulins and complement in these patients (5–7). Although the mechanisms by which severe injury induces immunosuppression are not yet clear, suppressor cells may be partially responsible as recently demonstrated by Miller and Baker (8).

To better understand the nature of immunoincompetency induced by trauma, we have developed an animal model in which mice are traumatized by surgical amputation of their right hind limbs. We have used this model in the present study to demonstrate that lymphocytes from these animals manifest significantly lessened immunologic reactivity. We have further attempted to identify the cause of this depressed cell-mediated immune responsiveness and have found macrophage-like suppressor cells in the spleens of these amputated mice.

METHODS

Animals. Male C57Bl/6 (H-2b), DBA/2 (H-2d), and CBA/J (H-2k) mice, age 8–12 wk, were used in this study. All strains were obtained from The Jackson Laboratory, Bar Harbor Maine.

Traumatization of animals. Animals were traumatized by surgical amputation of their right hind limbs. Mice were anesthetized with ether, the upper femur was broken with heavy forceps, and the limbs were excised with an electrosurgery. The wound was closed with autoclips.

Preparation of mouse lymphoid cells. Spleens were removed from mice at varying intervals after amputation. A single cell suspension was prepared by teasing the spleens apart and rinsing through Nos. 40 and 80 stainless steel mesh screens. These splenocytes were subsequently purified by Ficoll-Hypaque gradient centrifugation. Cells were washed three times and counted in a hemocytometer. The viability of these cells was always >98% as judged by trypan blue dye exclusion. Peritoneal exudate cells (PEC) were ob-

1 Abbreviations used in this paper: C, complement; CL, cytolytic lymphocytes; Con A, concanavalin A; FCS, fetal calf serum; GA, glass adherent; GNA, glass nonadherent;

Dr. Wu is a recipient of a grant from the Wellcome Travel Trust.

Received for publication 29 January 1980 and in revised form 11 April 1980.
tained by washing the peritoneal cavity with Hank's balanced salt solution (HBSS). We did not inject any peritoneal stimulant before harvesting PEC to avoid nonspecific activation of macrophages. Approximately 30–40% of PEC were esterase-positive cells as determined by cytochemical staining with α-naphthyl butyrate-esterase (9).

**Nylon wool column filtration.** To obtain a population rich in T cells, splenocytes purified by Ficoll-Hypaque gradient centrifugation were filtered through two successive nylon wool columns as described by Julius et al. (10). Approximately 300 mg of nylon wool (Leuko-Pak, Fenwal Inc., Ashland, Mass.) was packed into a 5-ml syringe. Before use, columns were incubated with RPMI 1640 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) at 37°C for 30 min. The columns were flushed with medium and 5 × 10^7 lymphocytes in 2 ml of RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS, Gibco Laboratories) were then placed on the columns. After incubation at 37°C for 30 min, effluent cells were collected by rinsing the columns with 10 ml of medium. These cells were subsequently placed on a second column and incubated at 37°C for an additional 30 min. The effluent cells from the second column were characterized as T lymphocytes because they no longer responded to lipopolysaccharide (LPS), whereas their responses to phytohemagglutinin (PHA) and concanavalin A (Con A) remained intact. Cells retained by the first nylon wool column were collected by repeatedly compressing the nylon wool. These cells had an increased LPS responsiveness, whereas both PHA and Con A responses were significantly decreased. Less than 0.1% of nylon wool-filtered cells were esterase-positive, whereas 3–5% of nylon wool retained cells were esterase-positive.

**Treatment of lymphocytes with anti-Thy 1.2 serum and complement (C).** T lymphocytes were depleted by treating splenocytes with anti-Thy 1.2 serum and C. Approximately 10^7 splenocytes were incubated in 1.5 ml of HBSS with monoclonal anti-Thy 1.2 antibody (5 × 10^−4 dilution) (New England Nuclear, Boston, Mass.) and Low-Tox guinea pig C (1/10 dilution) (Cedarlane Laboratories, Hicksville, N. Y.) at 37°C for 60 min. The cells were then washed three times. This treatment depleted T lymphocytes because both PHA and Con A responsiveness of these treated cells was destroyed, whereas the LPS response remained intact.

**Treatment of lymphocytes with anti-immunoglobulin (Ig) antibody and C.** Approximately 10^7 splenocytes were incubated in 1 ml of HBSS with 1/8 dilution of rabbit anti-mouse lg serum (Miles Laboratories Inc., Elkhart, Ind.) at 37°C for 30 min. 0.5 ml of a 1/5 dilution of C was then added and the cells were incubated for an additional 30 min. The cells were then washed and examined for their responsiveness to various mitogens. These treated cells were found to be functional T lymphocytes because they no longer responded to LPS, whereas both PHA and Con A responses remained intact.

**Collection of adherent cells with glass petri dishes.** Splenocytes were suspended in RPMI 1640 medium containing 10% FCS at a cell concentration of 5–10^6/ml. These cells were incubated in glass petri dishes at 37°C in a 10% CO2 atmosphere for 2–3 h. Cells not adhering to the dishes were carefully removed and designated as glass nonadherent (GNA) cells. After washing the dishes with warmed medium twice, glass adherent (GA) cells were collected by gently scraping the dishes with a rubber policeman. GA cells were predominantly macrophages, since >90% were esterase-positive cells, whereas the GNA population contained <2% esterase-positive cells.

**Removal of phagocytic cells with carbonyl iron and magnet.** Macrophages were also depleted by the carbonyl iron and magnet technique. Ficoll-Hypaque gradient purified splenocytes were suspended in carbonyl iron lymphocyte separator reagent (Technicon Instruments Corp., Tarrytown, N. Y.) at a cell concentration of 5 × 10^6/ml. After 20 min incubation at 37°C, cells were immediately diluted with 5 vol of HBSS. Phagocytic cells were removed by four successive treatments with a magnet. About 50% of the original cells remained after this procedure, and <1% were shown to be esterase-positive cells.

**Proliferative response of splenocytes to alloantigens.** Single cell suspensions were prepared from C57BL/6 and DBA/2 or in some instances CBA mice as previously described. Mouse erythrocytes were lysed by a 3-min exposure to a 0.83% Tris-NH4Cl solution. After being washed three times with HBSS, these splenocytes were suspended in RPMI 1640 medium containing 5% FCS, 2 mM L-glutamine (Gibco Laboratories), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Gibco Laboratories). C57BL/6 responder splenocytes together with various members of DBA/2 or CBA splenocytes that had been irradiated with 1,500 rad gamma radiation in 0.2 ml of medium were added to each well of a Falcon Microtest II culture plate (Falcon Labware, Div. of Becton, Dickinson & Co., Oxford, Calif.). The culture plates were incubated at 37°C, in a 10% CO2 and water-saturated atmosphere for 48 h. 1 μCi of [3H]thymidine (20 Ci/mmol sp act, New England Nuclear) was added to each well and cultures were continued for an additional 16 h. Cells were harvested by a Mash II automatic harvesting machine (Microbiological Associates, Walkersville, Md.). 2H-labeled DNA of the splenocytes was precipitated on a glass fiber filter after the cells were lysed with 5% trichloroacetic acid. Each sample was added to 5 ml of cocktail D (a mixture of 100 g naphthalene, 5 g of PPO, and 1 liter of 1,4-dioxane) and counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). The mean counts per minute (cpm) was obtained from triplicate cultures and the stimulation index (SI) was calculated by the following formula: SI = cpm of responder splenocytes co-cultured with irradiated stimulating splenocytes per cpm of responder splenocytes alone. The significance of the difference between the stimulation index of normal C57BL/6 splenocytes and that of splenocytes from traumatized C57BL/6 mice was determined by Student's t test.

**Induction of alloreactive cytolytic lymphocytes (CL) in mixed-lymphocyte cultures (MLC).** Splenocytes were prepared from mice as described. 10^7 C57BL/6 cells were incubated with 8 × 10^5 irradiated DBA/2 cells in 4 ml of RPMI 1640 medium containing 5% FCS, 2 mM L-glutamine, 10 mM HEPES (Gibco Laboratories), 0.4 mM 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.) and 100 U/ml of penicillin and 100 μg/ml of streptomycin. These MLC were performed in 16 × 125 mm glass culture tubes for 5 d at 37°C, in a 10% CO2 in air, water-saturated atmosphere. After incubation, cells were washed twice with HBSS and counted. Approximately 40% of the total original cells were viable at this time.

**Microcytotoxicity assay.** Mastocytoma cells, P815 (H-2b), maintained by serial intraperitoneal passage in DBA/2 mice were used as targets. After being washed once with HBSS, 5–7 × 10^4 P815 cells were incubated with 300 μCi 51Cr as sodium cromate (275 mCi/mg sp act, New England Nuclear) in 0.5 ml of HBSS at 37°C for 90 min. Cells were then washed

HBSS, Hanks’ balanced salt solution; LPS, lipopolysaccharide (E. coli 055:B5); L:T, lymphocyte to target cell ratio; MLC, mixed-lymphocyte culture; PEC, peritoneal exudate cells; PHA, phytohemagglutinin.

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three times and suspended in RPMI 1640 medium containing 10% FCS. 10⁶ of these ⁵¹Cr-labeled P815 cells in 0.05 ml were added to each well of a Linbro microtiter plate (IS-MVC-96S; Linbro Chemical Co., Hamden, Conn.). Effector C57BL/6 cells generated in 5-d MLC in 0.1 ml of medium were also added to each well at effector to target cell (L:T) ratios of 70:1, 35:1, and 17.5:1. Unless otherwise noted results were presented from cultures with an L:T ratio of 70:1.

Microtiter plates were centrifuged at 40 g for 5 min, and then incubated at 37°C for 3.5 h. After incubation, plates were centrifuged at 600 g for 10 min and 0.1 ml of culture medium was carefully removed and counted in a gamma counter (Packard Instrument Co.). The cytotoxicity presented as percent lysis was calculated from triplicate cultures by the following formula: percent lysis = \((E - S/M - S) \times 100\), where \(E\) = counts per minute of ⁵¹Cr from experimental cultures; \(S\) = counts per minute of ⁵¹Cr spontaneously released from labeled P815 cells; \(M\) = counts per minute of ⁵¹Cr released from labeled P815 cells that had been frozen and thawed three times. Statistical analysis was done by Student's \(t\) test.

RESULTS

Effect of trauma on lymphocyte proliferation in response to alloantigens. 10⁶ splenocytes prepared from normal C57BL/6 mice or from mice that had had their limbs amputated 2 d earlier, were co-cultured with various numbers of irradiated DBA/2 splenocytes in one-way MLC. After incubation, incorporated [³H]thymidine was measured and the stimulation index was subsequently calculated. As indicated in Fig. 1, the proliferative response to alloantigens was impaired when splenocytes were prepared from traumatized mice. A significant difference between normal splenocytes and splenocytes from traumatized mice was seen when they were co-cultured with 7.5 \(\times\) 10⁶ and 10⁶ DBA/2 mouse splenocytes (\(P < 0.05\) and \(<0.01\), respectively).

Effect of trauma on the induction of CL in vitro. In the next series of experiments, we compared the induction of CL in vitro in splenocytes from normal animals with that in splenocytes from animals whose limbs were amputated 2 d earlier. As shown in Table I, normal C57BL/6 splenocytes incubated with irradiated DBA/2 splenocytes for 5 d became cytolytic to P815 tumor cells. In contrast, splenocytes obtained from traumatized C57BL/6 mice were significantly less cytolytic to P815 cells after co-culture with DBA/2 splenocytes. These results clearly suggest that the ability of splenocytes from the traumatized animals to be converted to CL responding to alloantigens is reduced. This conclusion was supported by a subsequent experiment in which a wider range of L:T ratios (100:1 to 12.5:1) were examined. As indicated in Fig. 2, splenocytes from normal mice manifested significantly greater cytotoxicity than those from traumatized mice at all L:T ratios tested. This decreased capability of CL induction was not simply due to the effect of anesthesia since the cytotoxicity of splenocytes from mice that had had anesthesia with ether without amputation of the limbs was similar to that of control splenocytes (Table II).

Kinetics of depressed cellular immunity after trauma. A group of age-matched C57BL/6 mice were housed in the same cage. Three mice were chosen to

<table>
<thead>
<tr>
<th>Experiment</th>
<th>L:T</th>
<th>Normal*</th>
<th>Traumatized†</th>
<th>(P)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>70:1</td>
<td>46.5±2.9</td>
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<td>37.5±1.4</td>
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<td>17.5:1</td>
<td>29.3±0.3</td>
<td>8.5±0.8</td>
<td>&lt;0.001</td>
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<td>2</td>
<td>70:1</td>
<td>67.4±2.1</td>
<td>35.9±2.8</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>35:1</td>
<td>41.4±4.6</td>
<td>23.7±0.9</td>
<td>0.01</td>
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<tr>
<td></td>
<td>17.5:1</td>
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<td>16.5±0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>70:1</td>
<td>35.7±1.1</td>
<td>1.5±0.1</td>
<td>&lt;0.01</td>
</tr>
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<td>35:1</td>
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<td>1.0±0.6</td>
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</tr>
<tr>
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<td>17.5:1</td>
<td>13.7±1.7</td>
<td>0.3±1.0</td>
<td>&lt;0.01</td>
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</tbody>
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* Effector cells prepared from normal mice.
† Effector cells prepared from traumatized mice.

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be amputated each day. All animals were killed 8 d after the beginning of the study. Splenocytes were prepared from each group of mice individually and mixed with irradiated DBA/2 lymphocytes. After 5 d in MLC, cells were tested for cytotoxicity against P815 targets. As shown in Fig. 3, the capability of lymphocytes to be converted to CL was again impaired in mice whose limbs were amputated. Even when amputation was performed as short a time as 2 h before testing, a significant decrease in the generation of CL became detectable. This effect gradually disappeared beginning 2 d after amputation, but it remained significant through the 6th d. An over-shoot effect was seen when mice were amputated 8 d before testing.

We also performed a similar experiment in which two mice were anesthetized with ether without amputation each day. All mice were killed at day 7 and their splenocytes were harvested for testing for the CL induction in vitro. As demonstrated in Fig. 4, none of these splenocyte populations was shown to have impaired CL induction. In fact, a slight increase in CL activity was seen in those splenocytes obtained from mice that had had anesthesia 4–6 d previously. This study confirmed our previous observation (Table II) and further suggested that anesthesia with ether was not responsible for the decreased immune reactivity seen with splenocytes from traumatized animals.

**Characterization of the mechanism responsible for depressing the generation of CL in traumatized mice.** We subsequently performed a series of experiments in an attempt to characterize the mechanism responsible for the depressed ability to generate CL in the cells of traumatized mice. In the first experiment (Table III) CL generated from the splenocytes of

**Table II**

*Effect of Anesthesia with Ether on the Induction of CL in MLC*

<table>
<thead>
<tr>
<th>L:T</th>
<th>Normal*</th>
<th>Anesthetized†</th>
<th>P</th>
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<tbody>
<tr>
<td>Lysis±SEM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70:1</td>
<td>80.0±2.5</td>
<td>80.6±5.2</td>
<td>0.9</td>
</tr>
<tr>
<td>35:1</td>
<td>81.0±4.7</td>
<td>77.8±4.9</td>
<td>0.7</td>
</tr>
<tr>
<td>17.5:1</td>
<td>63.9±3.8</td>
<td>62.7±1.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Effector cells prepared from untreated mice.
† Effector cells prepared from mice anesthetized with ether 2 d earlier.

**Figure 2** Splenocytes were prepared from normal (○) or traumatized (□) C57BL/6 mice. After incubation with irradiated DBA/2 splenocytes for 5 d, these cells were tested for the cytotoxicity against ⁵¹Cr-labeled P815 target cells at L:T ratios of 100:1 to 12.5:1.

**Figure 3** A group of C57BL/6 mice were housed in the same cage. Three mice were chosen to be traumatized each day and all animals were killed 8 d after the beginning of this study. Splenocytes were prepared from these mice, incubated with irradiated DBA/2 splenocytes for 5 d in MLC, and then tested for their cytotoxicity. Splenocytes from normal mice were similarly tested and served as controls.

**Figure 4** A group of C57BL/6 mice were housed in the same cage. Two mice were chosen to be anesthetized with ether without amputation of the limbs. All mice were killed at day 7 and their splenocytes were harvested and tested for the induction of CL in MLC.
traumatized animals showed a significantly lessened cytotoxicity (percent lysis = 38.1) as compared to CL from the splenocytes of normal control mice (percent lysis = 50.0). However, this depressed generation of CL was abolished when splenocytes from traumatized animals were treated with carbonyl iron and a magnet (percent lysis = 65.1) or removal of GA cells (percent lysis = 61.6), suggesting that cells capable of phagocytosis and adhering to glass, presumably macrophages, were responsible for preventing the induction of CL in the splenocytes of traumatized mice. As anticipated, GA cells recovered from the petri dishes were not cytolytic (percent lysis = 28.0).

In a second experiment normal cells after being treated with iron/magnet and glass adherence procedures (GNA cells) did not significantly alter the cytolytic activity, thus ruling out the possibility that the techniques employed contributed to the results seen in the previous experiment. GA normal cells were incapable of generating CL.

In a third experiment splenocytes from traumatized mice again generated less CL activity in vitro (percent lysis = 37.7) than splenocytes from normal mice (percent lysis = 59.7). Removal of GA cells from trauma splenocytes increased activity (percent lysis = 64.9). However, admixing $5 \times 10^6$ and $10^6$ GA cells from traumatized mice with GNA cells of the same mice again significantly decreased the cytotoxicity of the GNA cells ($P < 0.01$ and $P < 0.05$, respectively). Addition of $10^6$ GA cells was ineffective.

Effect of spleen cells from traumatized animals on the proliferative response of normal mouse lymphocytes in ML C. We subsequently attempted to determine whether or not the apparent suppressor cell population in the spleens of traumatized mice would affect the proliferative response of normal mouse lymphocytes to alloantigens. $5 \times 10^6$ normal C57BL/6 mouse lymphocytes together with $5 \times 10^6$ CBA or DBA/2 mouse lymphocytes previously irradiated with 1,500 rad and $5 \times 10^6$ splenocytes prepared from normal or traumatized C57BL/6 mice were cultured for 2 d. 1 $\mu$Ci $[^3H]$thymidine was then added and the amount of $[^3H]$thymidine incorporated into DNA was measured 16 h later. The proliferative response was calculated as the SI as before. In the first experiment (Fig. 5, left), splenocytes from traumatized mice significantly decreased the capability of normal C57BL/6 mouse lymphocytes to proliferate in response to CBA mouse lymphocytes in vitro (group D) as compared to control cultures (group A; $P = 0.01$). GA cells prepared

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Effector cells</th>
<th>Percent lysis ± SEM</th>
<th>P*</th>
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<tr>
<td>1</td>
<td>Normal cells (control)</td>
<td>50.0±2.5</td>
<td>0.04</td>
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<tr>
<td></td>
<td>Trauma cells</td>
<td>38.1±2.2</td>
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</tr>
<tr>
<td></td>
<td>Iron/magnet-treated trauma cells</td>
<td>65.1±2.7</td>
<td>0.01</td>
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<tr>
<td></td>
<td>GNA trauma cells</td>
<td>61.6±1.5</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>GA trauma cells</td>
<td>28.0±1.7</td>
<td>0.001</td>
</tr>
<tr>
<td>2</td>
<td>Normal cells (control)</td>
<td>47.3±1.3</td>
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</tr>
<tr>
<td></td>
<td>Iron/magnet-treated normal cells</td>
<td>55.3±3.1</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>GNA normal cells</td>
<td>47.2±5.2</td>
<td>NS</td>
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<td></td>
<td>GA normal cells</td>
<td>26.9±2.0</td>
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<td>3</td>
<td>Normal cells (control)</td>
<td>59.7±1.9</td>
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<td></td>
<td>Trauma cells</td>
<td>37.7±0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>GNA trauma cells</td>
<td>64.9±3.1</td>
<td>NS</td>
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<tr>
<td></td>
<td>GNA trauma cells + $5 \times 10^6$</td>
<td>45.6±1.8</td>
<td>&lt;0.01 (NS)</td>
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<tr>
<td></td>
<td>GA trauma cells</td>
<td>55.2±1.8</td>
<td>NS (0.05)</td>
</tr>
<tr>
<td></td>
<td>GNA trauma cells + $1 \times 10^6$</td>
<td>63.3±1.9</td>
<td>NS (NS)</td>
</tr>
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</table>

* P values were determined by comparing cytolytic activity of trauma cells with that of normal control cells.

† P values in parentheses were determined by comparing cytolytic activity with that of GNA trauma cells without the addition of GA trauma cells (percent lysis = 64.9).
from traumatized mice further inhibited normal lymphocyte proliferation (group E; P < 0.01), whereas GNA cells from the same traumatized mice had no effect (group F). Furthermore, addition of GA cells (group B) or GNA cells (group C) prepared from normal mice had no effect. A similar observation was made in a second experiment in which DBA/2 mouse lymphocytes were used as stimulating cells (Fig. 5, right). The proliferative response of normal lymphocytes was again significantly suppressed in MLC when these cells were cocultured with unfractionated (group D; P < 0.01) or GA (group E; P < 0.001) splenocytes from traumatized animals, but not with GNA trauma cells (group F). Furthermore, GA and GNA splenocytes from normal mice were again shown to be without effect.

**Effect of suppressor cells from traumatized animals on the induction of CL in the splenocytes of normal mice.** We attempted to determine the effect of suppressor cells found in the spleens of traumatized animals on the ability of normal lymphocytes to generate CL in vitro. We prepared conventional MLC by mixing $10^7$ normal C57BL/6 splenocytes with $8 \times 10^6$ irradiated DBA/2 spleenocytes. Various numbers of trauma cells were also added to some cultures. After 5 d incubation, cells were examined for their cytotoxicity. As demonstrated in Fig. 6, addition of $10^6$ and $5 \times 10^6$ splenocytes from traumatized animals significantly decreased the capability of normal lymphocytes to become CL. GA cells purified from the splenocytes of traumatized mice showed a similar suppressive effect at lesser cell numbers. Furthermore, PEC harvested from the same mice were highly effective in preventing the generation of CL from normal splenocytes.

In further experiments presented in Table IV, the addition of unfractionated splenocytes from traumatized mice consistently caused suppression of CL induction in vitro. Again, this suppressive effect was completely abrogated when GA cells, shown to be highly suppressive, were removed (experiment 1). In contrast, both GNA and GA cells similarly prepared from spleens of normal mice had no effect. In the second experiment, the suppressive activity of splenocytes of traumatized mice was diminished by filtration of the cells twice through nylon wool columns. Cells retained by nylon wool were collected, tested and found to be suppressive of CL induction. Treatment of trauma splenocytes with anti-Thy 1.2 plus C and C alone did not alter the suppressive effect (experiment 3). Although suppressor cells treated with anti-Ig plus C showed a slightly lessened suppressive ac-

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**FIGURE 5**  $5 \times 10^6$ normal C57BL/6 mouse lymphocytes were cultured with $5 \times 10^6$ irradiated CBA (left) or DBA/2 (right) mouse lymphocytes. $5 \times 10^6$ additional unfractionated, GA and GNA splenocytes from normal C57BL/6 mice were added to cultures A, B, and C, respectively. The same number of unfractionated, GA, and GNA splenocytes prepared from traumatized mice were also added to cultures D, E, and F, respectively. Stimulation index was calculated from triplicate cultures. Bars indicate SEM.
cytes

After 5 d incubation, the cytotoxicity of the normal splenocytes was examined. GA cells isolated from trauma splenocytes were similarly examined (○). Cytotoxicity of CL from normal mice served as controls.

tivity, this was not significantly different from that of untreated suppressor cells (P = 0.06). Finally, additions of normal splenocytes fractionated on nylon wool columns or treated with various antisera and C to normal MLC did not inhibit the generation of CL, suggesting that the observations made in the previous experiments were not the result of the techniques used for purifying subsets of cells (experiment 4).

DISCUSSION

The findings reported in the present study are of practical interest because of the increasing body of evidence that deficiencies of immune responsiveness occur in traumatized patients and the probability that immunodeficiency in these patients is related to an increased susceptibility to life threatening sepsis. Ritzmann et al. (5) and Parker et al. (6) reported that after severe thermal burns and major operations patients had abnormally low levels of circulating Ig associated with an increased incidence of bacterial infection. Decreased immunoglobulin levels were similarly reported in traumatized animals by Alexander and Moncrief (11), and Cooper et al. (12). However, completely contradictory findings were reported by Rapaport and Bachvaroff (13), Markley et al. (14), and Kinnaeart et al. (15). Arturson and Fjellström (7) observed diminished serum complement levels in patients with burns and found that lethality of the burns was associated with lower complement levels. Alexander et al. established a positive correlation between abnormal neutrophil function and bacteremia in surgical patients (16). Christou et al. also reported that impaired neutrophil function was associated with anergy and susceptibility to infection in patients undergoing major surgery (17).

While antibody formation, complement activity, and neutrophil function are unquestionably important components of the host defense against sepsis, an intact cellular immune response is also apparently required for adequate resistance to at least some varieties of bacterial infection since clinical anergy, clearly a reflection of deficient T lymphocyte responsiveness, is associated with a grave prognosis and an increased risk of sepsis in surgically or accidentally traumatized patients (1, 2, 13, 18–20). This association suggests that abnormalities of cellular immunity detected in patients and experimental animals after trauma and burns have potential clinical significance.

It has been demonstrated that the survival of skin allografts is prolonged in patients who have severe burns and that the length of graft survival is directly related to the severity of the burns (21). Lymphoid cells obtained from rats subjected to burns or surgical injury are unable to manifest graft-vs.-host reactions in recipient rats differing at a minor histocompatibility locus (22). The incidence of tumor take (23, 24) and spontaneous metastases (25) is increased when tumor-injected animals are subjected to surgical operations. The number of T lymphocytes is significantly decreased after burns as measured by rosette formation with sheep erythrocytes (26). Blast transformation in response to PHA is impaired in lymphocytes obtained from postoperative patients as compared to those harvested before operation (27). Although lymphocytes from burned patients reportedly have an increased PHA responsiveness (28), both stimulating and responding capability are lessened when these lymphocytes are tested in MLC (29). Tumor-bearing mice previously subjected to burns failed to produce cytolytic effector cells capable of destroying specific tumor targets (30). In summary, these studies indicate that cellular immune responses are adversely affected by traumatic or thermal injury.

Evidence presented in this report adds further weight to this concept since we have clearly demonstrated that splenocytes harvested from mice that had been surgically traumatized showed a significantly lessened capacity to proliferate and to generate CL in response to alloantigens as compared to splenocytes harvested from normal mice. This immunoincompetency could be detected as soon as 2 h after trauma and persisted for 6 d. It was fully reversed when adherent and phagocytic cells were removed from the splenocytes. This finding in conjunction with the fact
that the restoration of adherent cells resulted in the return of suppression suggested that macrophages were responsible for the decreased immunologic reactivity seen in the traumatized animals. Furthermore, addition of splenocytes and PEC from traumatized mice suppressed the response of lymphocytes from normal mice to alloantigens, indicating further that suppressor cells were generated in mice after surgical trauma. The decreased CL induction in traumatized mice was not simply due to the effect of anesthesia since the cytotoxicity of splenocytes from mice that had been subjected to anesthesia with ether without amputation was not decreased.

Suppressive activity was completely abrogated when GA cells were removed from the splenocytes of traumatized mice. The GA cells were esterase-positive and were shown to be highly suppressive. In contrast, both GNA and GA cells from normal mice were inactive. Spleen cells from traumatized mice retained by nylon wool were active in inhibiting normal lymphocytes from responding to alloantigens, whereas nylon wool nonadherent cells were not. Finally, treatment with anti-Thy 1.2 and anti-Ig sera in the presence of C had no significant effect on the activity of the suppressor cells. Therefore, we conclude that macrophage-like cells that are Thy 1.2-negative, Ig-negative, esterase-positive, and capable of adhering to glass and nylon wool were responsible for the suppression seen in this study.

Although the mechanism by which trauma induces immunosuppression is not yet fully understood, several possibilities have been suggested. Our own laboratory (18), Munster et al. (2), and Ninneman et al. (31) have shown that serum obtained from patients after major surgery or severe burns significantly inhibited the blast transformation of normal lymphocytes in vitro in response to PHA. We (18) and Constantin (32, 33) reported that the suppressive factor(s) in trauma and burn serum was a polypeptide fraction with a mol wt < 10,000. We have further shown that the presence of such low molecular weight suppressive material in the serum is associated with clinical anergy in patients after major surgery (18).

Suppressor cells may also be responsible for the immunodeficiency caused by trauma as suggested by Munster in 1976 (34). However, direct evidence to support this hypothesis was not available until a recent

**TABLE IV**

*Characterization of Suppressor Cells from Traumatized Animals*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Action of cells to normal mouse MLC*</th>
<th>Percent lysis±SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (control)</td>
<td>62.5±2.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Unfractionated trauma splenocytes</td>
<td>49.3±0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>GNA trauma splenocytes</td>
<td>61.5±1.7</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>GA trauma splenocytes</td>
<td>0.4±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>GNA normal splenocytes</td>
<td>66.2±3.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>GA normal splenocytes</td>
<td>55.2±2.4</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>None (control)</td>
<td>70.1±3.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Unfractionated trauma splenocytes</td>
<td>55.3±1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Nylon wool-eluted trauma splenocytes</td>
<td>67.3±1.4</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Nylon wool-retained trauma splenocytes</td>
<td>52.7±1.6</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>None (control)</td>
<td>66.0±2.5</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Unfractionated trauma splenocytes</td>
<td>29.8±2.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Anti-Thy 1.2 + C-treated trauma</td>
<td>31.6±1.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>splenocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-Ig + C-treated trauma splenocytes</td>
<td>41.7±1.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
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<td>C-treated trauma splenocytes</td>
<td>29.5±2.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>None (control)</td>
<td>76.7±5.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Nylon wool-eluted normal splenocytes</td>
<td>72.4±7.3</td>
<td>NS</td>
</tr>
<tr>
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<td>Nylon wool-retained normal splenocytes</td>
<td>80.2±4.9</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Anti-Thy 1.2 + C-treated normal splenocytes</td>
<td>81.9±4.5</td>
<td>NS</td>
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<tr>
<td></td>
<td>Anti-Ig + C-treated normal splenocytes</td>
<td>73.2±6.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>C-treated normal splenocytes</td>
<td>85.3±7.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Splenocytes prepared from mice whose limbs were amputated 2 d earlier were fractionated by adherence to glass petri dishes or nylon wool columns (two cycles), or by treating with anti-Thy 1.2 or anti-Ig sera and C. 2 x 10⁶ nylon-treated and 5 x 10⁶ anti-sera treated cells were added to normal conventional MLC.
study in which Miller and Baker (8) were able to abolish normal human MLC reactions by adding lymphoid cells from severely burned patients. The present findings also indicate that suppressor cells were in large measure responsible for the suppression of cellular immunity seen in surgically traumatized animals. However, the suppressor cells in our system appeared to be macrophages.

There is a growing body of evidence suggesting that macrophages as well as lymphocytes may act as suppressor cells. Perkins and Makinodan (35) reported that production of humoral antibody by lymphocytes was decreased by the presence of macrophages. Parkhouse and Dutton (36) inhibited lymphocyte proliferation in response to antigens and mitogens by adding macrophages to the cultures. A similar suppressive effect by macrophages was demonstrated by Fernbach et al. (37) in MLC. Kirchner et al. (38) observed that macrophages obtained from spleens of mice bearing tumors induced by Moloney’s virus inhibited the DNA synthesis of syngeneic normal lymphocytes in response to PHA. Their observation was later confirmed by Veit and Feldman (39) and Elgert and Farrar (40) in various tumor-host systems. Antigen specific proliferative responses of lymphocytes from patients with chronic schistosomiasis were suppressed and this was due to the presence of suppressor monocytes in these patients (41, 42). The depression of PHA-induced blast transformation in lymphocytes from patients with advanced malignancy was again partially accounted for by the presence of suppressive monocytes as reported by Zembala et al. (43), Goodwin et al. (44), and Quan and Burtin (45). Although macrophages have been long known to function as accessory cells potentiating various reactivities of lymphocytes, results from the present study and from these mentioned above further suggest that macrophages may play an opposite and equally important role in immunologic regulation.

ACKNOWLEDGMENTS

The authors are grateful to Doctors G. S. Pinkus and E. L. Milford for their assistance throughout this study. We also wish to thank K. Collins, I. Saporoschetz, and D. DePina for their technical as well as secretarial assistance.

This study was supported by U. S. Public Health Service grant GM 26016 and Army contract DAMD 17-76-C-6076.

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