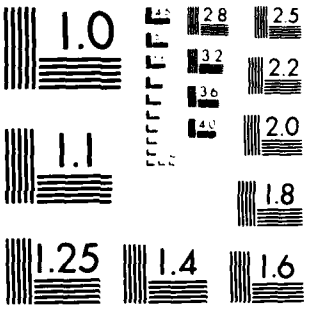
AD-A135 671 INVESTIGATION OF IMMUNOREGULATORY ALPHAGLOBULIN (IRA) 1/
IN SHOCK AND TRAUMA(U) BRIGHAM AND WOMEN'S HOSPITAL
BOSTON MA J A MANNICK JUL 80 DAMD17-76-C-6076

UNCLASSIFIED F/G 6/5 NL

END
DATE FILMED
1 · 84
DTIC



"AD _____"

(1)

AD-A135 671

Investigation of Immunore ulatory Alphaglobulin (IRA) in Shock and Trauma

Annual Progress Report

John A. Mannick, M.D.

July, 1980

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick
Frederick, Maryland 21701

Contract No. DAMD 17-76-C-6076

Brigham and Women's Hospital
Boston, Massachusetts 02115

(Unclassified)

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A135 671	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Investigation of Immunoregulatory Alphaglobulin (IRA) in Shock and Trauma		5. TYPE OF REPORT & PERIOD COVERED Annual Report 7/1/79 - 6/30/80
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) John A. Mannick, M.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD 17-76-C-6076
9. PERFORMING ORGANIZATION NAME AND ADDRESS Brigham and Women's Hospital Boston, Massachusetts 02115		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS02.00.003
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701		12. REPORT DATE July 1980
		13. NUMBER OF PAGES 28
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Trauma	Cellular immunity
Burns	Immunosuppression
Lymphocyte activation	Sepsis

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The major accomplishment of the past year of research ~~supported by contract DAMD 17-76-C-6076~~ was the demonstration that a suppressed (by more than 50%) response to PHA was associated with grave prognosis. We further demonstrated that the suppressed PHA response of the lymphocytes of these individuals was caused by the presence of circulating suppressor cells. Nearly all patients with burn injuries of greater than 35% body surface area were found to have circulating low molecular weight immunosuppression factors in the serum. –

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

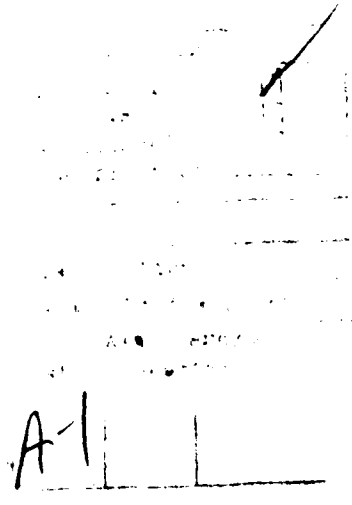
SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

However the presence of such factors could not be related to the survival of the patients. At a dose of approximately 5 mg per animal, a low molecular weight fraction obtained from suppressive serum of burn patients and patients who had undergone surgical trauma was shown to suppress the ability of normal mice to resist infection with Listeria monocytogenes. The same fraction recovered from the serum of normal volunteers had no effect.

FOREWORD

(a) Citations of commercial organizations and trade names in this report do not constitute an office Department of the Army endorsement or approval of the products or services of these organizations.

(b) For the protection of human subjects the investigator(s) have adhered to policies of applicable Frederal Law 45CFR46.



Annual Progress Report - During the past year we have published the study of patients undergoing major cardiovascular surgery reported in the previous progress report (see attached reprint). Among these major surgical patients, anergy and suppressive serum in the postoperative period were significantly associated with postoperative infectious complications and with days spent in the hospital. Further investigation of these individuals failed to show that fraction 11 is active in suppression of cellular immunity in mice when injected in vivo. However, this failure may be due to the extremely small quantities of fraction 11 available for study. Analysis of fraction 11 by the Hoffman LaRoche Institute of Molecular Biology has indicated that fraction 11 eluted from filter paper following high voltage electrophoresis contains a number of amino acids and other impurities from the filter paper. When these are eliminated by high pressure liquid chromatography a small amount of a pure molecular species was obtained. Because of extremely small quantities, hydrolysis could only be performed once. This yielded one unique and not previously identified amino acid and several common amino acids.

During the past year we have continued to study the serum of patients having undergone major cardiovascular surgery and the serum from 19 burn patients, the majority of whom had burns of greater than 35% of body surface area. Attempts to recover suppressive material from these sera by DEAE cellulose chromatography and gel filtration on G25 Sephadex columns, as mentioned in the previous progress report, has consistently shown that the majority of suppressive activity is contained in a low molecular weight fraction, peak III, from G25 Sephadex columns. Starting sera (in 10% concentration) suppressed activation by PHA of normal human peripheral blood lymphocytes by 50% or more. Peak III from such sera ordinarily suppressed human lymphocyte activation by 50% or more at concentrations of 100 micrograms per ml or less. High voltage electrophoresis of Peak III from such sera, however, did not consistently show the presence of fraction 11. This was particularly true when burn sera were studied. While some sera contained fraction 11 and fraction 11 was consistently suppressive of lymphocyte activation in vitro when present, activity was also found in the mid neutral range by high voltage electrophoresis in some sera.

It is clear that further work will be necessary before the homogeneous molecular species responsible for the suppressive activity of the heterogeneous Peak III can be identified and characterized. We are currently processing a large serum pool from 12 burn patients with suppressive sera in an attempt to resolve this problem.

During the past year burn patients have been the major focus of our clinical studies. As noted in last year's progress report we had determined at the beginning of this year's contract support that in 10 burn patients with greater than 30% body surface burn, anergy to skin test antigens was correlated with the presence of suppressive serum as defined by the ability of the serum in 10% concentration to suppress by 50% or more the stimulation of normal human peripheral blood lymphocytes to PHA in vitro. This correlation remains statistically significant at the conclusion of the present research year after the addition of 9 more burn patients to the study. It is still clear, however, that while not all burn patients with suppressive serum are anergic, nearly all anergic patients have suppressive serum.

Serum immunosuppression developed in 16 of the 19 burn patients studied at some time during their hospitalization and 13 of these patients developed a systemic infection. It was not, however, possible to relate the time of appearance of more than 50% serum immunosuppressive activity with existing or subsequent infection. The absence or presence of serum immunosuppression at some time in the post burn course did not correlate with survival (Table I). The concentration of E-rosette forming cells in the peripheral blood of burn patients correlated with the presence of serum immunosuppressive activity ($r = 0.57$, $p = 0.01$). Twelve patients demonstrated very low levels of E-rosette forming cells in the peripheral blood (mean = 6.4% rosettes) but following 6 washes of the peripheral blood lymphocyte population the E-rosette forming cell concentration rose to a mean of 16.5%. This suggests that some T cells were inhibited from forming E-rosettes by a loosely bound suppressive substance. The percentage of E-rosettes also correlated with the PHA response of the peripheral blood lymphocytes ($r = 0.47$, $p = 0.05$).

Most burn patients did not demonstrate an impaired response of the peripheral blood lymphocytes to PHA stimulation in vitro. However, a depressed PHA response was associated with severe infection and high mortality (Table I and II). Four of the 7 patients who manifested this finding died. Conversely, no burn patient died without manifesting severe depression of the peripheral blood lymphocyte response to PHA. The presence of circulating suppressor leukocytes as evidenced by their ability to suppress the response of normal human lymphocytes to PHA was studied serially in 7 patients (27 samples) and correlated with depression of PHA-induced blastogenesis ($r = 0.074$, $p < 0.001$).

These studies suggest that the presence of circulating low molecular weight immunosuppressive substances is an almost invariable consequence of a major burn injury. Although this circulating immunosuppressive material may inhibit host resistance to infection many patients who manifest this finding survive. However, the development of 50% or more suppression of response of peripheral blood lymphocytes to PHA and the appearance of circulating suppressor leukocytes is associated with a grave prognosis. A summary of this work will be published in the Surgical Forum (reprint enclosed). A more complete report of this work is under preparation.

Investigations of the biological effects of low molecular weight serum suppressive material (G25 Sephadex peak III) from trauma and burn patients have been carried out in animal systems. Peak III material from burn patients, from normal individuals and from patients who developed suppressive serum following aortic aneurysm replacement was injected into groups of 10 A/Jax mice which were then challenged with an LD 25 dose of Listeria monocytogenes organisms, usually 5×10^4 organisms. The peak III from burn patients at 5 mg per mouse induced a 90% mortality. Control mice manifested a 10% mortality. Control mice manifested a 10% mortality. Peak I from the same burn serum also yielded a 10% mortality, and Peak III administered without Listeria did not produce any mortality. Similarly, Peak III from aneurysm patients induced a 60% mortality with a control mortality of 20%, Peak I from aneurysm patients induced a 10% mortality. Peak III from pooled normal serum yielded a 20% mortality, the same as in control mice. The whole pooled normal serum protein yielded a 30% mortality. We believe that these experiments offer convincing evidence that the circulating low molecular weight immunosuppressive material from burn and trauma patients suppresses the resistance to sepsis.

We also initiated experiments during the past year to determine the effect of Peak III material on the induction of suppressor cells in mice. Spleens from mice previously injected with 5 mg of Peak III from burn patients were able to suppress the PHA response of normal mouse spleen cells in a graded dose response fashion.

Finally, during the past year we have developed an animal model in which to study the induction of impaired cellular immune responsiveness following trauma in order to study this phenomenon under more controlled conditions than is possible in man and especially in order to study in the future the potential usefulness of various therapeutic interventions directed towards abrogating the inhibition of cellular immunity seen under these conditions. Splenocytes from C57BL mice traumatized by amputation of the right hind limbs under general anesthesia were consistently shown to have a significantly (by Students $\underline{t}$ test) diminished capacity to proliferate in response to alloantigens and to form alloreactive cytolytic cells in mixed lymphocyte culture (Table III).

TABLE I

Burn Patients

	Survivors (14 patients)	Deaths (5 patients)
Mean % Serum Suppression*	72.0%	69.4%
Mean Reduction in PHA Blastogenesis*	30.8%	82.4%

* mean of greatest level of suppression measured in each patient

TABLE II

Burn Patients

	No. Patients	Mean Hospital Stay	Deaths	Systemic Infection	Serum Suppression of PHA Response >50%	Impaired Rosette Formation <15%	Lack of PHA Response (<50% Normal)
35% Chance Survival*	8	165 days	5	7	5/8	3/5	4/5
70-100% Chance Survival*	11	45 days	0	7	11/11	8/9	3/11

Determined by prior experience at our center

TABLE III

Comparison of Cytolytic Activity After MLC of Lymphocytes from Normal Animals with those from Traumatized Animals

Experiment	L:T *	Normal ‡ (% Lysis ± SEM)	Trauma §	P
1	70:1	46.5 ± 2.9	18:1 ± 0.3	<0.001
	35:1	37.5 ± 1.4	13.6 ± 1.4	<0.001
	17.5:1	29.3 ± 0.3	8.5 ± 0.8	<0.001
2	70:1	67.4 ± 2.1	35.9 ± 2.8	<0.001
	35:1	41.4 ± 4.6	23.7 ± 0.9	0.01
	17.5:1	26.0 ± 1.9	16.5 ± 0.4	<0.01
3	70:1	35.7 ± 1.1	1.5 ± 0.1	<0.01
	35:1	21.8 ± 0.8	1.0 ± 0.6	<0.001
	17.5:1	13.7 ± 1.7	0.3 ± 1.0	<0.01

* Lymphocyte to target cell ratio.

‡ Effector cells prepared from at least 3 normal mice.

§ Effector cells prepared from at least 3 traumatized mice. (amputation 2 days earlier)

TABLE IV

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

L:T *	Normal †	Anesthetized §	P
	(% Lysis ± SEM)		
70:1	80.0 ± 2.5	80.6 ± 5.2	0.9
35:1	81.0 ± 4.7	77.8 ± 4.9	0.7
17.5:1	63.9 ± 3.8	62.7 ± 1.9	0.8

* Lymphocyte to target cell ratio.

† Effector cells prepared from at least 3 untreated mice.

§ Effector cells prepared from at least 3 mice anesthetized with ether two days earlier.

TABLE V

Characterization of Suppressor Cells from Traumatized Animals

Experiment	Addition of cells* to normal mouse MLC	% lysis ± SEM	P
1	None (control	62.5 ± 2.2	-
	Unfractionated trauma splenocytes	49.3 ± 0.4	< 0.01
	GNA trauma splenocytes	61.5 ± 1.7	NS+
	GA trauma splenocytes	0.4 ± 0.8	< 0.00
	GNA normal splenocytes	66.2 ± 3.4	NS
	GA normal splenocytes	55.2 ± 2.4	NS
2	None (control)	70.1 ± 3.2	-
	Unfractionated trauma splenocytes	55.3 ± 1.0	< 0.01
	Nylon wool-eluted trauma splenocytes	67.3 ± 1.4	NS
	Nylon wool-retained trauma splenocytes	52.7 ± 1.6	0.01
3	None (control)	66.0 ± 2.5	-
	Unfractionated trauma splenocytes	29.8 ± 2.8	< 0.00
	Anti-Thy 1.2 + C-treated trauma splenocytes	31.6 ± 1.2	< 0.00
	Anti-Ig + C-treated trauma splenocytes	41.7 ± 1.2	< 0.01
	C-treated trauma splenocytes	29.5 ± 2.2	< 0.00
4	None (control)	76.7 ± 5.1	
	Nylon wool-eluted normal splenocytes	72.4 ± 7.3	NS
	Nylon wool-retained normal splenocytes	80.2 ± 4.9	NS
	Anti-Thy 1.2 + C-treated normal splenocytes	81.9 ± 4.5	NS
	Anti-Ig + C-treated normal splenocytes	73.2 ± 6.2	NS
	C-treated normal splenocytes	85.3 ± 7.8	NS

* Splenocytes prepared from mice whose limbs were amputated 2 days earlier were fraction by adherence to glass Petri dishes or nylon wool columns (2 cycles), or by treating wi anti Thy 1.2 or ant-Ig sera and C . $2x10^6$ nylon-treated and $5x10^6$ anti-sera treated c were added to normal conventional MLC.

DISTRIBUTION LIST

4	copies	USAMRDC (SGRD-RMS) Fort Detrick Frederick, MD 21701
12	copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDA Cameron Station Alexandria, VA 22314
1	copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20014
1	copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234
4	copies	Commander Letterman Army Institute of Research (LAIR) Bldg. 1110 ATTN: Dr. J. Ryan Neville Presidio of San Francisco, CA 94129

2135

IMMUNOLOGY

INDUCTION OF SUPPRESSOR CELLS BY TRAUMA. B. S. Wang, E. H. Heacock*, and J.A. Mannick, Harvard Medical School and Peter Bent Brigham Hospital, Boston, MA 02115.

Trauma is often accompanied by a loss of immunocompetency which may contribute to morbidity and mortality from infectious complications. In order to better understand the nature of the immunoincompetency induced by trauma, we undertook the present study. C57BL/6 mice were traumatized by surgical amputation of their right hind limbs. Splenocytes were prepared from these mice two days after amputation. The immunoreactivity of these cells was tested in an in vitro system in which alloreactive cytolytic lymphocytes (CL) were generated. Splenocytes from traumatized mice were consistently shown to have a diminished capacity to form CL. This incompetency was detected from two hours to six days after surgical trauma and was completely reversed by removing phagocytic cells from the cultures. Furthermore, addition of these trauma splenocytes to mixed lymphocyte cultures from normal mice prevented normal lymphocytes from being sensitized to alloantigens, suggesting the presence of suppressor cells. The suppressor cells were found to adhere to glass and to nylon wool columns. They were resistant to treatment with anti-thy 1.2 and anti-Ig sera in the presence of complement. Therefore, results suggest that a thy 1.2-negative, Ig-negative cell population capable of adhering to glass and nylon wool, presumably macrophages, was responsible for inhibiting the response of lymphocytes to alloantigens in traumatized animals. (Supported by USPHS GM26016-01.)

Reprinted from American College of Surgeons 1980 Surgical Forum Volume XXXI.

CHAPTER III – *Infections, Trauma, and Burns*

RELATIVE CLINICAL IMPORTANCE OF SERUM SUPPRESSOR FACTORS AND CELLULAR SUPPRESSION IN MAJOR BURNS

John H. N. Wolfe, MB, FRCS,
Nicholas E. O'Connor, MD, FACS,
Anthony E. Young, McH, FRCS, Inna Saporoschetz, AB,
and John A. Mannick, MD, FACS

SYSTEMIC INFECTION is the leading cause of morbidity and mortality in burned patients. Both cellular immune suppression (1) and serum immunosuppressive factors (2) may inhibit host resistance to the bacteria invariably present in burn wounds. The relative clinical importance of these immune responses is poorly understood. We have therefore studied 19 patients (aged 20 to 84 years) with full-thickness burns and related their clinical course to the following immunologic functions: serum suppression of normal PHA blastogenesis, E-rosette formation, the PHA response of nylon wool nonadherent cells, and the presence of nylon wool nonadherent suppressor T cells.

MATERIALS AND METHODS

The clinical course of the patients was monitored and their chance of survival was assessed from the Brigham Hospital burn data by probit analysis. The patients were then divided into two groups: less than 35% and greater than 35% chance of survival. During the course of the illness, peripheral venous blood samples were tested for immunosuppressive activity in vitro. Fourteen normal volunteers served as controls. Nonadherent T cells were recovered by eluting them from a nylon wool column. Their viability was greater than 90% with the trypan blue exclusion technique. The blastogenic response of these cells to phytohemaglutinin (PHA) was tested after two days incubation. The results were expressed as counts/minute of the incorporated ^{3}H-thymidine and related to the response of normal control cells. A stimulation of less than 50% of normal was considered significant depression. The patients' cells were also incubated with normal cells and PHA to assess the ability of the cells to suppress

From the Department of Surgery, Harvard Medical School, Peter Bent Brigham Hospital, Boston.

the normal lymphocyte response. E-rosette concentration was measured, and less than 15% of rosette-forming cells was considered abnormal. Serum samples were studied for their ability to inhibit normal PHA-induced lymphocyte proliferation. Sera were added to the culture medium of microtiter plates in 10% and 20% concentrations. Controls included cultures with no addition of serum and those with 10% and 20% pooled normal serum.

RESULTS

Serum immunosuppression developed in 16 of 19 patients during their illness, and 13 of these patients developed a systemic infection. The time of immunosuppression could not be related to existing or subsequent infection, however, and two severely burned patients with systemic infection did not have suppressive serum. The presence of serum immunosuppression did not correlate with survival.

The concentration of E-rosette-forming cells correlated with the presence of serum immunosuppression ($r = 0.57$, $P = 0.01$) after a single cell wash but not after six washings. Twelve patients failed to form E-rosettes when the cells were washed once (mean = 6.4% rosette formation) but after six washes the E-rosette cell concentration rose to a mean of 16.5%. This suggests that some T cells were inhibited from forming E-rosettes by a loosely bound suppressive substance. E-rosette concentration also correlated with the PHA response of the cells ($r = 0.47$, $P = 0.05$).

Failure of the peripheral blood lymphocyte response to PHA was uncommon and was associated with severe infections and a high mortality. Of the seven patients with this finding, four died, one developed a candida endocarditis (despite having only a 40% burn), and the other two had a protracted hospital stay and system infections (84 and 60 days hospitalization for 35% burns). No patient died without severe depression of the lymphocyte response to PHA.

In 27 samples the PHA response was tested after a single cell washing and after six washes, and improved more than 50% after six washes. In 11 samples, however, there was no difference between washing the cells three and six times. After the single wash, serum factors may still affect the PHA response but the failure to improve blastogenesis after repeated washings suggests that the suppression is intrinsic and not caused by an inhibitor loosely attached to the cell surface. The results of PHA response after three and six washes were therefore used to measure the degree of suppression of the lymphocytes.

The presence of suppressor T cells was studied serially in seven patients (27 samples) and correlated with the failure of PHA blastogenesis ($r = 0.074$, $P < 0.001$).

Serial studies in 13 patients showed that serum suppressive factors developed during the first week after the burn and in the more severely

Table 1–Clinical outcome related to immune response

	No. patients	Mean hospital stay (days)	Deaths	Systemic infection	Serum suppression of PHA response > 50%	Impaired rosette formation < 15%	Lack of cellular PHA response (< 50% normal)
< 35%							
Chance survival	8	165	5	7	5/8	3/5	4/5
35%–100%							
Chance survival	11	45	0	7	11/11	8/9	3/11

burned patients the T cell response to PHA also became subnormal, but a T cell response of less than 50% of normal was uncommon.

CONCLUSION

Serum immunosuppression is an almost invariable consequence of a moderate or major burn injury. Although this immunosuppression may inhibit host resistance to infection, many of these patients survive. However, the development of suppressed blastogenesis of peripheral blood lymphocytes and the appearance of circulating suppressor T cells is associated with a grave prognosis (Table 2).

Table 2–Prognosis after appearance of circulating suppressor T cells

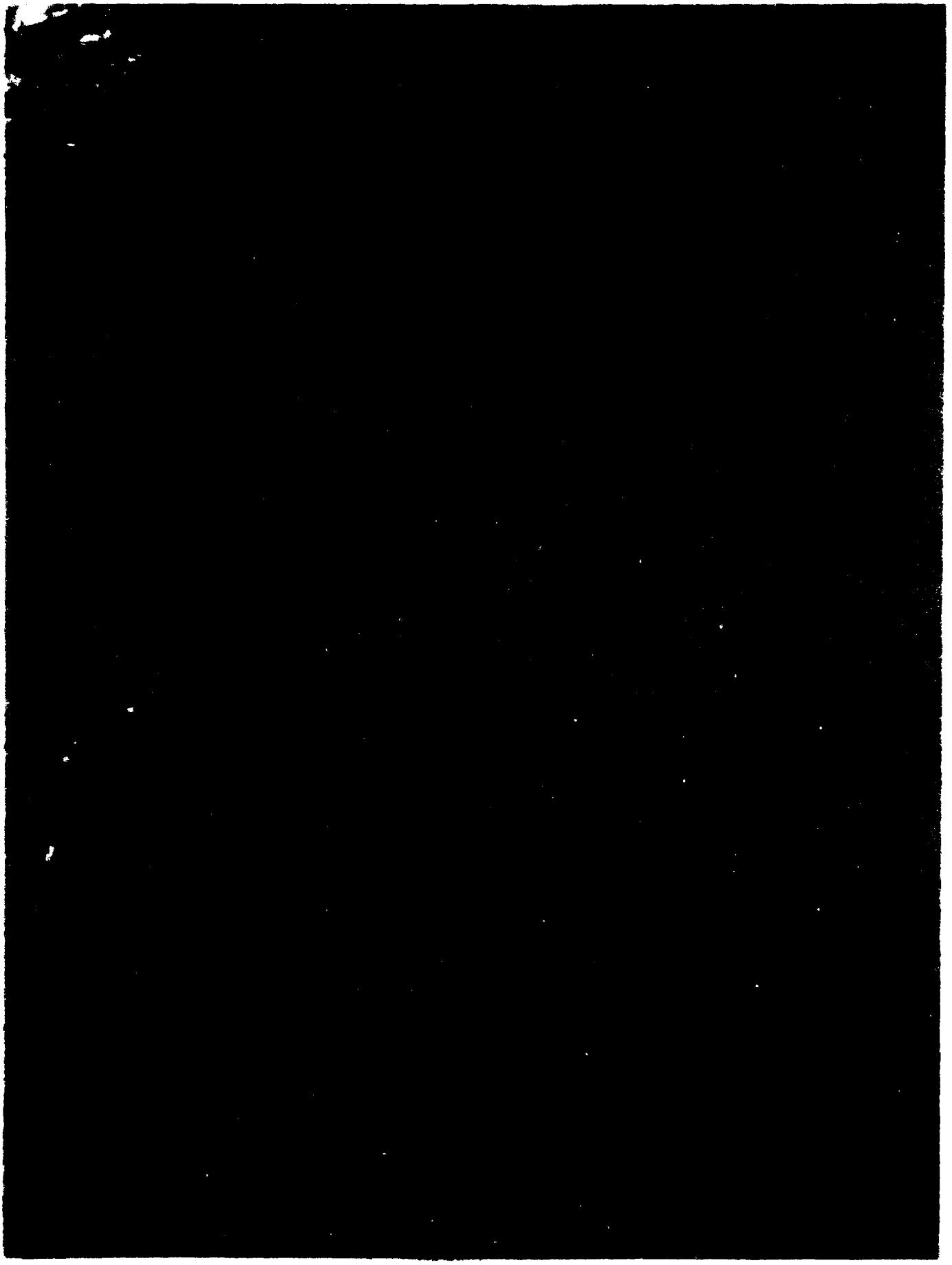
	Survivors (N = 14) (%)	Deaths (N = 5) (%)
Mean % serum suppression	72.0	69.4
Mean reduction in PHA blastogenesis	30.8	82.4

Mean of greatest level of suppression measured in each patient.

REFERENCES

1. Miller CL, Baker CC: Changes in lymphocyte activity after thermal injury. J Clin Invest 63:202-210, 1979

2. Constantian MB: Association of sepsis with an immunosuppressive polypeptide in the serum of burn patients. Ann Surg 188:209-215, 1978



Generation of Suppressor Cells in Mice after Surgical Trauma

BOSCO SHANG WANG, ELOISE H. HEACOCK, ANDREW V. O. WU, and JOHN A. MANNICK,
Department of Surgery, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT 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/6J (H-2^b), DBA/2J (H-2^d), and CBA/J (H-2^k) 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 electrocautery. 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)[1] were ob-

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.

[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;

tained by washing the peritoneal cavity with Hanks' 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 Ig 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 $5–10 \times 10^6$/ml. These cells were incubated in glass petri dishes at 37°C, in a 10% CO_2 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-NH_4Cl 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 numbers of DBA/2 or CBA splenocytes that had been irradiated with 1,500 rad of 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., Oxnard, Calif.). The culture plates were incubated at 37°C, in a 10% CO_2 and water-saturated atmosphere for 48 h. 1 μCi of [^{3}H]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.). ^{3}H-labeled DNA of the splenocytes was precipitated on a glass fiber filter after the cells were lysed with 5% trichloracetic 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 8×10^6 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% CO_2 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-2^d), maintained by serial intraperitoneal passage in DBA/2 mice were used as targets. After being washed once with HBSS, $5–7 \times 10^6$ P815 cells were incubated with 300 μCi ^{51}Cr 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.

three times and suspended in RPMI 1640 medium containing 10% FCS. 10^4 of these ^{51}Cr-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) × 100, where E = counts per minute of ^{51}Cr from experimental cultures; S = counts per minute of ^{51}Cr spontaneously released from labeled P815 cells; M = counts per minute of ^{51}Cr 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. 10^6 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 [^{3}H]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 7.5×10^5 and 10^6 DBA/2 mouse splenocytes ($P < 0.05$ and <0.01, respectively).

Effects 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).

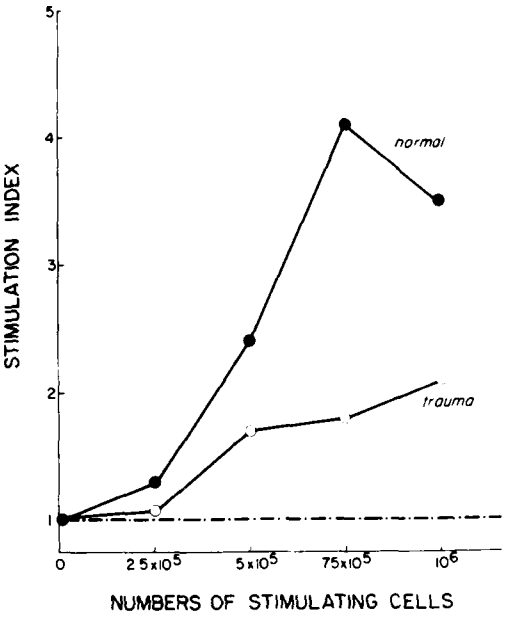


FIGURE 1 10^6 splenocytes prepared from normal (●) or traumatized (○) C57BL/6 mice were cocultured with 2.5×10^5–10^6 irradiated DBA/2 splenocytes in a one-way MLC. After incubation, incorporated [^{3}H]thymidine was measured and the stimulation index was subsequently determined (Methods).

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 I
Comparison of Cytolytic Activity after MLC of Lymphocytes from Normal Animals with those from Traumatized Animals

Experiment	L:T	Normal*	Trauma†	*P*
		% lysis ± SEM		
1	70:1	46.5±2.9	18.1±0.3	<0.001
	35:1	37.5±1.4	13.6±1.4	<0.001
	17.5:1	29.3±0.3	8.5±0.8	<0.001
2	70:1	67.4±2.1	35.9±2.8	<0.001
	35:1	41.4±4.6	23.7±0.9	0.01
	17.5:1	26.0±1.9	16.5±0.4	<0.01
3	70:1	35.7±1.1	1.5±0.1	<0.01
	35:1	21.8±0.8	1.0±0.6	<0.001
	17.5:1	13.7±1.7	0.3±1.0	<0.01

* Effector cells prepared from normal mice.
† Effector cells prepared from traumatized mice.

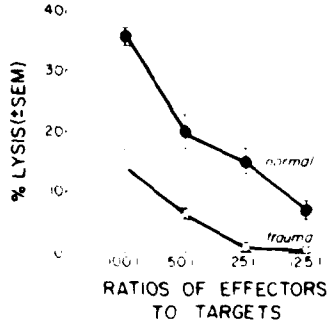


FIGURE 2 Splenocytes were prepared from normal (●) or traumatized (○) C57BL/6 mice. After incubation with irradiated DBA/2 splenocytes for 5 d, these cells were tested for the cytotoxicity against ^{51}Cr-labeled P815 target cells at E:T ratios of 100:1 to 12.5:1.

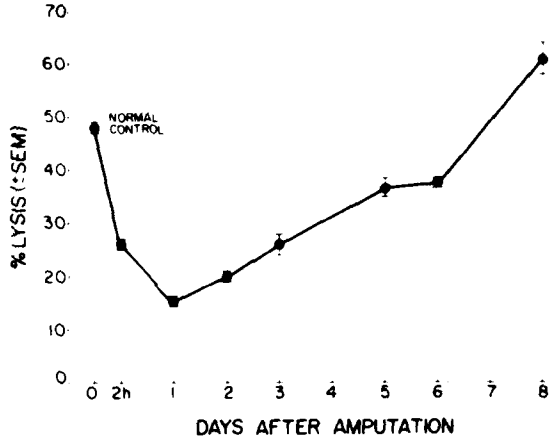


FIGURE 3 A group of C57BL/6 mice were housed in the same cage. Three mice were chosen to be traumatized each day and all animals were killed 8 d after the beginning of this study. Splenocytes were prepared from these mice, incubated with irradiated DBA/2 splenocytes for 5 d in MLC, and then tested for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

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.

TABLE II
Effect of Anesthesia with Ether on the Induction of CL in MLC

E:T	Normal*	Anesthetized‡	P
	lysis ± SEM		
70:1	80.0±2.5	80.6±5.2	0.9
35:1	81.0±4.7	77.8±4.9	0.7
17.5:1	63.9±3.8	62.7±1.9	0.8

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

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

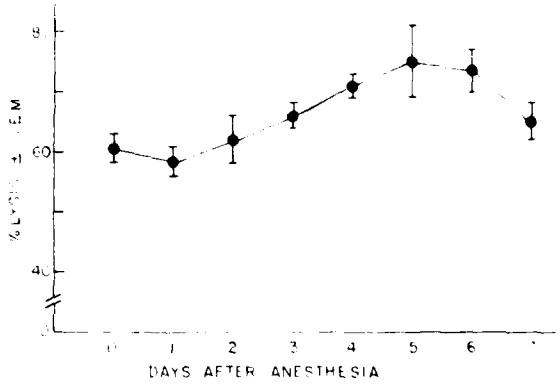


FIGURE 4 A group of C57BL/6 mice were housed in the same cage. Two mice were chosen to be anesthetized with ether without amputation of the limbs. All mice were killed at day 7 and their splenocytes were harvested and tested for the induction of CL in MLC.

TABLE III
Characterization of the Cell Type Responsible for Suppressing the Induction of CL in Traumatized Animals

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

* *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).

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 5×10^6 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 10^5 GA cells was ineffective.

Effect of spleen cells from traumatized animals on the proliferative response of normal mouse lymphocytes in MLC. 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. 5×10^5 normal C57BL/6 mouse lymphocytes together with 5×10^5 CBA or DBA/2 mouse lymphocytes previously irradiated with 1,500 rad and 5×10^5 splenocytes prepared from normal or traumatized C57BL/6 mice were cultured for 2 d. 1 μCi [^{3}H]thymidine was then added and the amount of [^{3}H]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

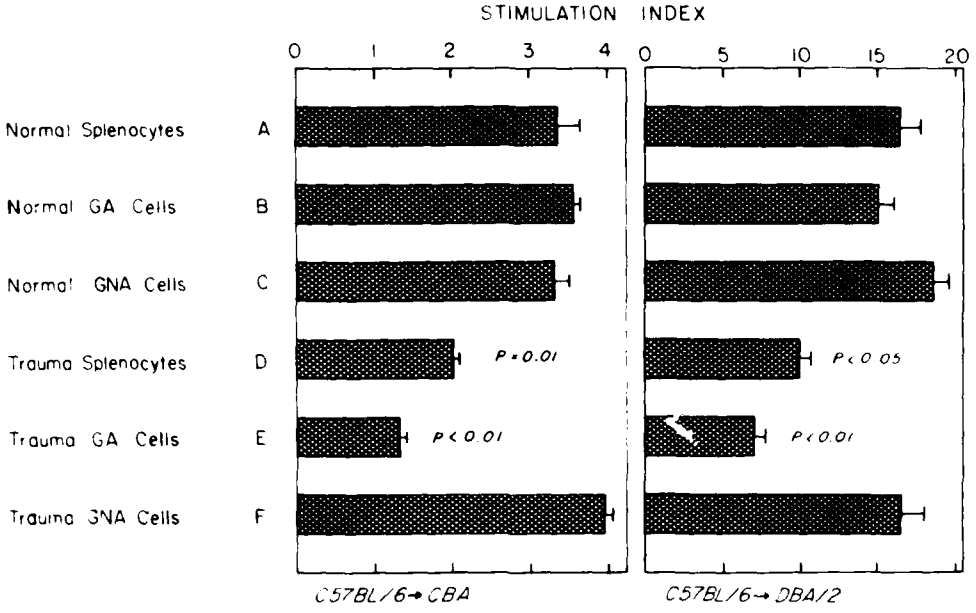


FIGURE 5 5×10^5 normal C57BL/6 mouse lymphocytes were cultured with 5×10^6 irradiated CBA (left) or DBA/2 (right) mouse lymphocytes. 5×10^5 additional unfractionated, GA and GNA splenocytes from normal C57BL/6 mice were added to cultures A, B, and C, respectively. The same number of unfractionated, GA, 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.

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 8×10^6 irradiated DBA/2 splenocytes. 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* 5×10^6 *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-

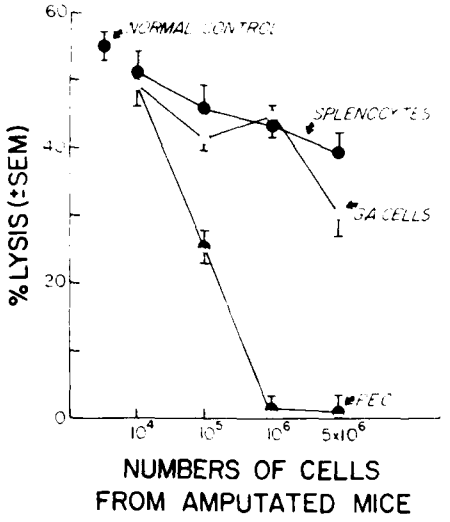


FIGURE 6 Various numbers of splenocytes (●) and PEC (▲) obtained from traumatized mice were added to normal MLC. After 5 d incubation, the cytotoxicity of the normal splenocytes was examined. GA cells isolated from trauma splenocytes were similarly examined (○). Cytotoxicity of CL from normal mice served 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 Kinnaert 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

TABLE IV

Characterization of Suppressor Cells from Traumatized Animals

Experiment	Addition of cells to normal mixed MLC*	Percent lysis ± SEM	P
1	None—control	62.5 ± 2.2	—
	Unfractionated trauma splenocytes	49.3 ± 0.4	0.01
	GNA trauma splenocytes	61.5 ± 1.7	NS
	GA trauma splenocytes	0.4 ± 0.8	0.001
	GNA normal splenocytes	66.2 ± 3.4	NS
	GA normal splenocytes	53.2 ± 2.4	NS
2	None—control	70.1 ± 3.2	—
	Unfractionated trauma splenocytes	55.3 ± 1.0	0.01
	Nylon wool-eluted trauma splenocytes	67.3 ± 1.4	NS
	Nylon wool-retained trauma splenocytes	52.7 ± 1.6	0.01
3	None—control	66.0 ± 2.5	—
	Unfractionated trauma splenocytes	29.8 ± 2.8	< 0.001
	Anti-Thy 1.2 + C-treated trauma splenocytes	31.6 ± 1.2	< 0.001
	Anti-Ig + C-treated trauma splenocytes	41.7 ± 1.2	< 0.01
	C-treated trauma splenocytes	29.5 ± 2.2	< 0.001
4	None—control	76.7 ± 5.1	
	Nylon wool-eluted normal splenocytes	72.4 ± 7.3	NS
	Nylon wool-retained normal splenocytes	80.2 ± 4.9	NS
	Anti-Thy 1.2 + C-treated normal splenocytes	81.9 ± 4.5	NS
	Anti-Ig + C-treated normal splenocytes	73.2 ± 6.2	NS
	C-treated normal splenocytes	85.3 ± 7.8	NS

* 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. 2×10^6 nylon-treated and 5×10^6 anti-sera treated cells were added to normal conventional MLC.

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 Nimeman 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 Constantian (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

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 Fembach 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 Burton (45). Although macrophages have been long known to function as accessory cells potentiating various reactivities of lymphocytes, results from the present study and from those mentioned above further suggest that macrophages may play an opposite and equally important role in immunologic regulation.

ACKNOWLEDGMENTS

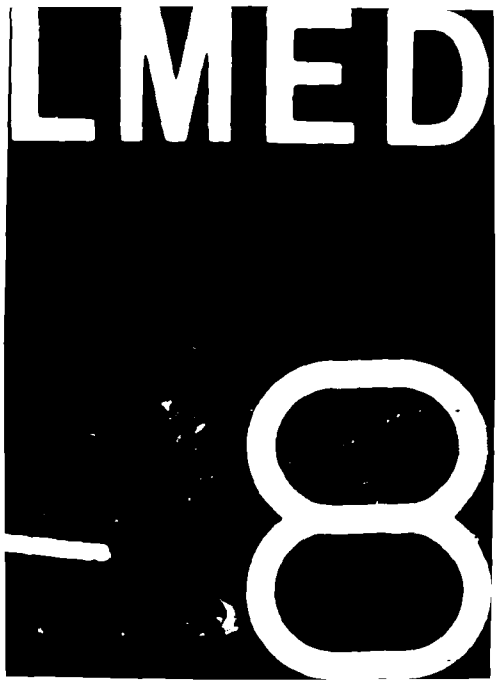
The authors are grateful to Doctors G. S. Pinkus and E. L. Milford for their assistance throughout this study. We also wish to thank K. Collins, I. Saporoschetz, and D. DePina for their technical as well as secretarial assistances.

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

REFERENCES

1. Pietsch, J. B., J. L. Meakins, and L. D. MacLean. 1977. The delayed hypersensitivity response: application in clinical surgery. *Surgery.* **82:** 349–355.
2. Munster, A. M., K. Eurenius, R. M. Katz, L. Canales, F. D. Foley, and R. F. Mortensen. 1973. Cell-mediated immunity after thermal injury. *Ann. Surg.* **177:** 139–143.
3. McRipley, R. J., and D. W. Garrison. 1965. Effect of burns in rats on defense mechanisms against *Pseudomonas aeruginosa*. *J. Infect. Dis.* **115:** 159–170.
4. Alexander, J. W., M. Hegg, and W. A. Altemeier. 1968. Neutrophil function in selected surgical disorders. *Ann. Surg.* **168:** 447–458.
5. Ritzmann, S. E., D. L. Larson, C. McClung, S. Alston, D. Falls, and A. S. Goldman. 1969. Immunoglobulin levels in burned patients. *Lancet.* **1:** 1152–1153.
6. Parker, D. J., J. W. Cantrell, R. B. Karp, R. M. Stroud, and S. B. Digerness. 1972. Changes in serum complement and immunoglobulins following cardiopulmonary bypass. *Surgery.* **71:** 824–827.
7. Arturson, G., and K. E. Fjellstrom. 1966. Complement-active serum protein in clinical burns. *In* Research in Burns. A. B. Wallace and A. B. Wilkinson, editors. E & S Livingstone, Ltd., Edinburgh. 401.
8. Miller, C. L., and C. C. Baker. 1979. Changes in lymphocyte activity after thermal injury. The role of suppressor cells. *J. Clin. Invest.* **63:** 202–210.
9. Li, C. Y., L. T. Yam, and W. H. Crosby. 1972. Histochemical characterization of cellular and structural elements of the human spleen. *J. Histochem. Cytochem.* **20:** 1049–1058.
10. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3:** 645–649.
11. Alexander, J. W., and J. A. Monteriel. 1966. Alterations of the immune response following severe thermal injury. *Arch. Surg.* **93:** 75–83.
12. Cooper, A. J., J. M. Irvine, and A. R. Turnbull. 1974. Depression of immunological responses due to surgery. A model in the guinea pig. *Immunology.* **27:** 393–399.
13. Rapaport, F. T., and R. J. Bachvaroff. 1976. Kinetics of humoral responsiveness in severe thermal injury. *Ann. Surg.* **184:** 51–59.
14. Markley, K., E. Smallman, and G. Evans. 1967. Antibody production in mice after thermal and tourniquet trauma. *Surgery.* **61:** 896–903.
15. Kinnaert, P., A. Mahieu, and N. Van Geertruyden. 1978. Stimulation of antibody synthesis induced by surgical trauma in rats. *Clin. Exp. Immunol.* **32:** 243–252.
16. Alexander, J. W., J. D. Stinnett, C. K. Ogle, J. D. Ogle, and M. J. Morris. 1979. A comparison of immunologic profiles and their influence on bacteremia in surgical patients with a high risk of infection. *Surgery.* **86:** 94–104.
17. Christou, N. V., and J. L. Meakins. 1979. Neutrophil function in anergic surgical patients: neutrophil adherence and chemotaxis. *Ann. Surg.* **190:** 557–564.
18. McLoughlin, G. A., A. V. Wu, I. Saporoschetz, R. Nimberg, and J. A. Mannick. 1979. Correlation between anergy and a circulating immunosuppressive factor following major surgical trauma. *Ann. Surg.* **190:** 297–304.
19. Meakins, J. L., J. B. Pietsch, O. Bubenick, R. Kelly, H. Rode, J. Gordon, and L. D. MacLean. 1977. Delayed hypersensitivity: indicator of acquired failure of host defenses in sepsis and trauma. *Ann. Surg.* **186:** 241–250.
20. Rapaport, F. T., F. Milgrom, K. Kano, B. Gesner, A. C. Solowey, P. Casson, H. I. Silverman, and J. M. Converse. 1968. Immunologic sequelae of thermal injury. *Ann. N. Y. Acad. Sci.* **150:** 1004–1008.
21. Chambler, K., and J. R. Batchelor. 1969. Influence of defined incompatibilities and area of burn on skin-homograft survival in burned subjects. *Lancet.* **1:** 16–18.
22. Munster, A. M., K. Eurenius, R. F. Mortensen, and A. D. Mason. 1972. Ability of splenic lymphocytes from injured rats to induce a graft-versus-host reaction. *Transplantation.* **14:** 106–108.

23. Saba, T. M., and T. G. Antikatzides. 1976. Decreased resistance to intravenous tumor-cell challenge during reticuloendothelial depression following surgery. *Br. J. Cancer* **34**: 381–389.
24. Munster, J. J. 1963. Decreased resistance to tumor cells after stress followed by increased resistance. *Proc. Soc. Exp. Biol. Med.* **113**: 377–379.
25. Lewis, M. R., and W. H. Cole. 1958. Experimental increase of lung metastases after operative trauma. Amputation of limb tumor. *Arch. Surg.* **77**: 621–626.
26. Neilan, B. A., L. Taddeini, and R. G. Strate. 1977. T lymphocyte rosette formation after major burns. *JAMA (J. Am. Med. Assoc.)* **238**: 493–496.
27. Park, S. K., J. I. Brody, R. A. Wallace, and W. S. Blakemore. 1971. Immunosuppressive effect of surgery. *Lancet* **1**: 53–55.
28. Mahler, D., and J. R. Batchelor. 1971. Phytohaemagglutinin transformation of lymphocytes in burned patients. *Transplantation*. **12**: 409–411.
29. Sakai, H., J. C. Daniels, G. A. Beathard, S. R. Lewis, J. B. Lynch, and S. E. Ritzmann. 1974. Mixed lymphocyte culture reaction in patients with acute thermal burns. *J. Trauma*. **14**: 53–57.
30. Markley, K., E. T. Smallman, and L. A. Lawrence. 1977. The effect of thermal trauma in mice on cytotoxicity of lymphocytes. *Proc. Soc. Exp. Biol. Med.* **154**: 72–77.
31. Ninnemann, J. L., J. C. Fisher, and T. L. Wachtel. 1979. Thermal injury-associated immunosuppression: occurrence and *in vitro* blocking effect on post recovery serum. *J. Immunol.* **122**: 1736–1741.
32. Constantian, M. B. 1978. Association of sepsis with an immunosuppressive polypeptide in the serum of burn patients. *Ann. Surg.* **188**: 209–215.
33. Constantian, M. B., J. D. Menzoian, R. N. Nimberg, K. Schmid, and J. A Mannick. 1977. Association of a circulating immunosuppressive polypeptide with operative and accidental trauma. *Ann. Surg.* **185**: 73–79.
34. Munster, A. M. 1976. Post-traumatic immunosuppression is due to activation of suppressor T cell. *Lancet*. **1**: 1329–1330.
35. Perkins, E. H., and T. Makinodan. 1965. The suppressive role of mouse peritoneal phagocytes in agglutinin response. *J. Immunol.* **94**: 765–777.
36. Parkhouse, R. M. E., and R. W. Dutton. 1966. Inhibition of spleen cells DNA synthesis by autologous macrophages. *J. Immunol.* **97**: 663–669.
37. Fernbach, B. R., H. Kirchner, and R. B. Herberman. 1976. Inhibition of the mixed lymphocyte culture by peritoneal exudate cells. *Cell. Immunol.* **22**: 399–403.
38. Kirchner, H., A. Muchmore, T. M. Chused, H. T. Holden, and R. B. Herberman. 1975. Inhibition of proliferation of lymphoma cells and T lymphocytes by suppressor cells from spleens of tumor-bearing mice. *J. Immunol.* **114**: 206–210.
39. Veit, B. C., and J. D. Feldman. 1976. Altered lymphocyte functions in rats bearing syngeneic Moloney sarcoma tumors. II. Suppressor cells. *J. Immunol.* **117**: 655–660.
40. Elgert, K. D., and W. L. Farrar. 1978. Suppressor cell activity in tumor-bearing mice. I. Dualistic inhibition by suppressor T lymphocytes and macrophages. *J. Immunol.* **120**: 1345–1353.
41. Todd, C. W., R. W. Goodgame, and D. G. Colley. 1979. Immune responses during human schistosomiasis mansoni. V. *Suppression of* schistosome antigen-specific lymphocyte blastogenesis by adherent phagocytic cells. *J. Immunol.* **122**: 1440–1446.
42. Ottesen, E. A. 1979. Modulation of the host response in human schistosomiasis. I. Adherent suppressor cells that inhibit lymphocyte proliferative responses to parasite antigens. *J. Immunol.* **123**: 1639–1644.
43. Zeuthala, M., B. Mytar, T. Popiela, and G. L. Asherson. 1977. Depressed *in vitro* peripheral blood lymphocyte response to mitogens in cancer patients. The role of suppressor cells. *Int. J. Cancer*. **19**: 605–613.
44. Goodwin, J. S., R. P. Messner, A. D. Bankhurst, G. T. Peake, J. H. Saiki, and R. C. Williams. 1977. Prostaglandin-producing suppressor cells in Hodgkin's disease. *N. Engl. J. Med.* **297**: 963–968.
45. Quan, P. C., and P. Burtin. 1978. Demonstration of non-specific suppressor cells in the peripheral lymphocytes of cancer patients. *Cancer Res.* **38**: 288–296.


LMED
8