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IN SHOCK AND TRAUMA(U) PETER BENT BRIGHAM HOSPITAL
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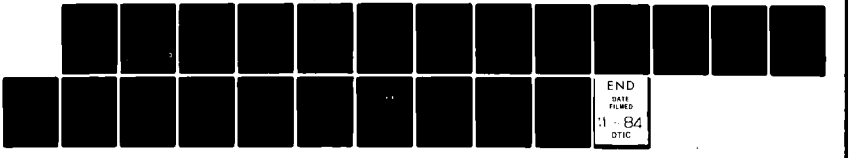

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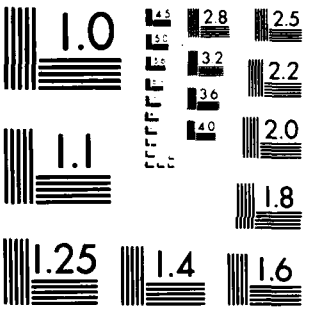

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Investigation of Immunoregulatory Alphaglobulin (IRA) in Shock and Trauma

Annual Progress Report

John A. Mannick, M.D.

June 1979

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The major accomplishment of the past year of research supported by contract DAMD 17-76-C-6076 was the characterization of the substance chiefly responsible for the immunosuppressive activity appearing in the serum of patients following major trauma and burns. Pooled serum from such patients was subjected to DEAE cellulose chromatography and further fractionated by gel filtration on G25 Sephadex. A low molecular weight fraction was found to contain the majority of the suppressive activity as determined by its ability to suppress PHA stimulation of normal human peripheral blood lymphocytes in tissue culture.

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It was also demonstrated that, at a dose of approximately 5 mg per animal, this low molecular weight fraction suppressed the plaque forming cell response to sheep erythrocytes in mice by more than 50%. The low molecular weight suppressive fraction was further fractionated by preparative high-voltage electrophoresis in distilled water and acetic acid. Individuals ninhydrin positive moieties were eluted from filter paper and recovered by lyophilization. These fractions were tested for suppressive activity in vitro and the majority of the activity was found in a highly basic fraction, fraction 11. This highly basic molecular species was not recovered from similarlu processed serum from patients who had undergone minor surgical trauma or from normal volunteers.

FOREWORD

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Annual Progress Report - During the past year we have completed evaluation of a series of patients undergoing major cardiovascular surgery. None of these individuals was malnourished pre-operatively and none had cancer. All individuals showed normal reactivity to skin testing with recall antigens pre-operatively and none had serum suppressive of normal human lymphocyte activation in tissue culture as defined by the ability of patient's serum in 10% concentration to suppress by 50% or more the response of normal human lymphocytes to stimulation by 3 different doses of PHA. As controls, we studied 15 patients who underwent minor operative procedures, ordinarily inguinal herniorraphy, under general anesthesia. Of the 31 patients who underwent major cardiovascular surgery, 14 developed suppressive serum by the 3rd post-operative day and of these 14 patients 11 became anergic to skin test antigens to which they had previously been reactive. Of the 17 patients who did not develop suppressive serum in the post-operative period, only 2 became anergic. The correlation between anergy and suppressive serum in the post-operative period in this patient group was highly significant. (See attached reprint) By the 28th post-operative day all patients who had undergone major cardiovascular surgery had recovered normal skin test reactivity and had lost suppressive activity in the serum. None of the patients who underwent minor surgical procedures developed suppressive serum or became anergic at any time in the post-operative period.

Among the major surgery patients, anergy and suppressive serum in the post-operative period were not associated with the number of blood transfusions administered in the peri-operative period nor the duration of anesthesia. However, they were significantly associated with post-operative infectious complications ($p < 0.05$) and with the days spent in the hospital post-operatively ($p < 0.01$).

In an attempt to determine the nature of the suppressive activity in the serum of these major cardiovascular surgery patients, a pooled sample of serum from 8 of these individuals was subjected to DEAE cellulose chromatography and the initial active peaks were then further fractionated by gel filtration on G25 Sephadex columns. A low molecular weight fraction, Peak III, was found to contain the majority of the suppressive activity as determined by its ability to suppress PHA stimulation of normal human lymphocytes in tissue culture. We also found that, at a dose of approximately 5 mg per mouse, Peak III suppressed the plaque forming cell response to sheep red blood cells in mice by more than 50%. High molecular weight fractions from the same pooled serum had no effect. G25 Peak III was further fractionated by a method of preparative high voltage electrophoresis in distilled water and acetic acid, pH 3.5. Individual ninhydrin positive moieties were eluted from filter paper and recovered by lyophilization. These fractions were tested for suppressive activity in vitro and the majority of the activity was found in the highly basic fraction, fraction 11. This highly basic molecular species was not recovered from a similarly processed sample of serum from patients who had undergone minor surgery or from normal volunteers (Figure 1).

Pure fraction 11 has been sent for analysis to the Hoffman LaRoche Institute of Molecular Biology. Results of gas liquid chromatography and mass spectrometry are not yet available. However, by high pressure liquid chromatography,

fraction 11 is found to be a pure molecular species. Since fraction 11 is homogeneous by both high voltage electrophoresis and high pressure liquid chromatography we believe we have recovered at least one molecule that has marked immunosuppressive activity as measured by in vitro inhibition of human T lymphocyte activation. Since this molecule is not found in detectable quantities in a similar sample of normal serum or in the serum of patients who have undergone minor operative trauma and since it appears to account for the majority, but certainly not all, of the immunosuppressive activity found in the serum of patients who have undergone major surgical trauma we believe that further investigation of this molecule is clearly warranted.

Preliminary results in our own laboratory with acid hydrolysis of fraction 11 have yielded amino acids and a basic component which migrates on high voltage electrophoresis between lysine and the known polyamines (Figure 2). We believe, therefore, that fraction 11 is likely to be a polypeptide containing a molecule with many free amino groups, perhaps a previously undescribed polyamine or a highly substituted amino acid. Since the estimated molecular weight of fraction 11 is approximately 1,000 daltons or slightly less it seems likely that fraction 11 could be commercially synthesized when its structure is known. Such an eventuality would, of course, greatly simplify detailed investigation of the immunological activity of this material and the development of a radioimmunoassay.

While we have not yet shown that pure fraction 11 is active in vivo in an animal species, the starting material from which fraction 11 was obtained, i.e. G25 Peak III, is clearly suppressive in the mouse of the immune response to a T dependent antigen in a manner similar to IRP fractions previously prepared in our laboratory from huge quantities of Cohn fraction IV obtained from commercial fractionation of human plasma. (See attached manuscript) This is the first demonstration to our knowledge of in vivo immunosuppressive activity of a serum fraction obtained from traumatized patients.

In six patients who had undergone major cardiovascular surgery, we looked at E-rosette formation by peripheral blood lymphocytes and found that 4 out of the 6 patients demonstrated a significant decrease in E-rosette formation (defined as 50% or more) as compared with simultaneously studied normal volunteers. Lymphocytes from these patients were then washed 6 times in tissue culture medium and tested again for E-rosette formation. E-rosettes increased after washing significantly (50% or more) in 3 of the patients. The material washed from the lymphocytes was then processed by filtration on an Amicon UM-05 ultrafilter and was recovered by lyophilization. This material was then dissolved in distilled water and chromatographed on G25 Sephadex columns. Low molecular weight material from the columns contained the majority of suppressive activity and, in fact, by preparative high voltage electrophoresis has been demonstrated to contain fraction 11. This fraction is also highly suppressive of T lymphocyte activation when tested in vitro as was fraction 11 obtained from the serum of the same patients.

During the past year, we have also begun to study burn patients as a separate subgroup of trauma patients. Ten burn patients, all with greater than 30% second and third degree burns were skin tested for hypersensitivity responsiveness to standard recall antigens and were sensitized to dinitrochlorobenzene (DNCB). Tests were performed on 29 occasions and were compared on each occasion with

the ability of the patient's serum in 10% concentration to suppress normal human lymphocyte stimulation by PHA in tissue culture. On 12 occasions the serum from the burn patients was found to be suppressive of PHA stimulation and on all but 2 of these occasions the patients were anergic to all skin test antigens. On 17 occasions the patients' serum was non-suppressive of PHA stimulation and on all but 2 of these occasions the patients were normallly reactive to skin test antigens. The correlation between suppressive serum and anergy determined simultaneously in these burn patients is highly significant ($p < 0.0005$) as shown in Table I. Seriously burned patients as a group appeared to have diminished E-rosette formation by their peripheral blood lymphocytes. As demonstrated in Table II the patients with 50% or more dimunition in E-rosette formation, as compared with simultaneously studied normal volunteers, were likely to have serum (drawn simultaneously) that was suppressive of PHA stimulation. In 6 of the 15 instances the E-rosette formation was significantly increased (> 50%) after washing the lymphocytes thoroughly in tissue culture medium in vitro. The material recovered by washing these lymphocytes and those from normal volunteers and patients undergoing minor surgical procedures is currently being compared for suppressive activity.

Finally the peripheral blood lymphocytes of burn patients have been studied for their response to PHA stimulation at a range of doses in tissue culture. Lymphocyte stimulation was considered suppressed when the responses, measured in CPM of H_3-thymidine incorporation, was < 50% of simultaneously studied normal volunteers. From the results of 24 studies on 11 seriously burned patients summarized in Table III, it is apparent that suppressive serum did not correlate at all with the ability of peripheral blood lymphocytes studied on the same day to respond to PHA stimulation in tissue culture. However, those patients who did demonstrate a diminished PHA response all had suppressive serum. The effect of washing the peripheral blood lymphocytes in tissue culture medium on their subsequent response to PHA stimulation, as shown in Table IV, also did not demonstrate any particular pattern. Both those patients who had normal PHA responsiveness and those with diminished responsiveness demonstrated increased lymphocyte responsiveness after in vivo washing in about 1/3 of instances. Control lymphocytes from normal volunteers increased PHA responsiveness after washing much less frequently.

This ongoing study of burn patients, therefore, has demonstrated conclusively that anergy in these patients is associated with serum significantly suppressive of T lymphocyte activation. It also appears likely that suppressive serum is correlated with diminished E-rosette formation by peripheral blood lymphocytes in these same individuals and that in nearly half those patients with diminished E-rosette formation the percentage of E-rosettes is increased after thorough washing of the lymphocytes in vitro. A correlation between the clinical course of the burn patients and the levels of E-rosettes, and the presence or absence of suppressive serum and the presence or absence of delayed hypersensitivity responsiveness is currently underway.

TABLE I

Correlation of Suppressive Serum with Anergy in 10 Burn Patients Tested on 29 Occasions.

Patients' Serum	Skin Test Positive	Skin Test Negative
Suppressive - 12	2	10
Non-Suppressive - 17	15	2

$Chi^2 = 12.05$

$p < 0.0005$

TABLE II

Diminished E-Rosette Formation of Lymphocytes From 10 Burn Patients Studied on 15 Occasions.

E-Rosette Diminished	Suppressive Serum	E-Rosettes Increased After 6 In Vitro Washes
15	14	6

TABLE III

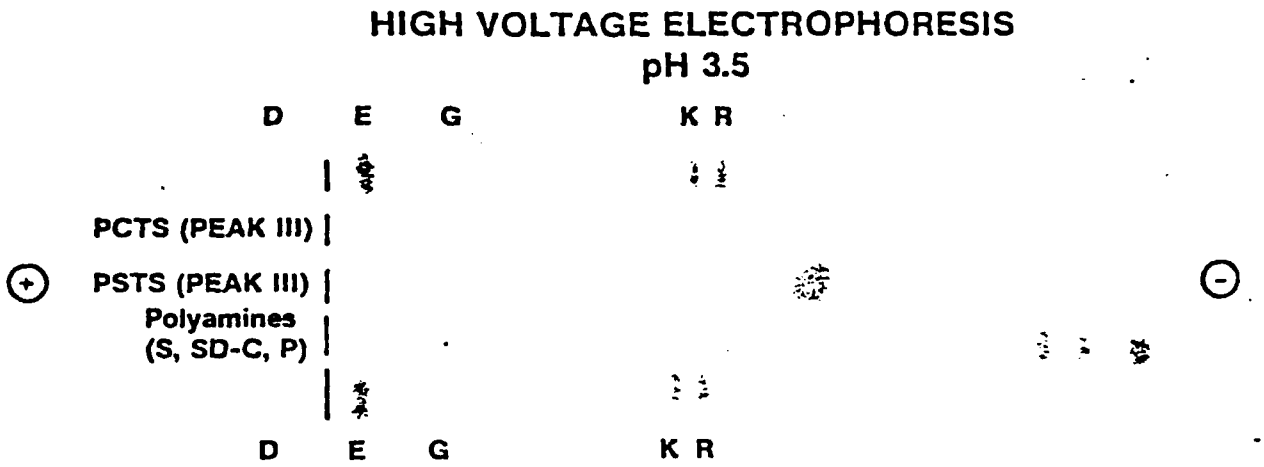
PHA Stimulation of Peripheral Blood Lymphocytes From 11 Burn Patients Studied on 24 Occasions.

Patients' Serum	Diminished PHA Response	Normal Response
Suppressive - 19	6	13
Non-Suppressive - 5	0	5

TABLE IV

Effect of Washing 6 Times In Vitro on
PHA Response of Lymphocytes From Burn Patients

Initial Response	Increased After Washing	Not Increased After Washing
Diminished - 6	2	4
Normal - 18	5	13
Control - 20	2	18



Immunosuppressive Fractions Isolated From Pooled Trauma Sera by Gel Filtration

Figure 1. High-voltage electrophoresis of pooled serum from 8 patients who developed anergy after major cardiovascular surgery (PSTS) and of pooled serum from patients who underwent inguinal herniorrhaphy under general anesthesia (PCTS). Ninhydrin stain was used. Fraction 11 is the ninhydrin positive moiety, slightly more basic than the lysine marker (R). However, Fraction 11 is not as basic as the known polyamines--spermine, spermidine, cadaverine and putrescine.

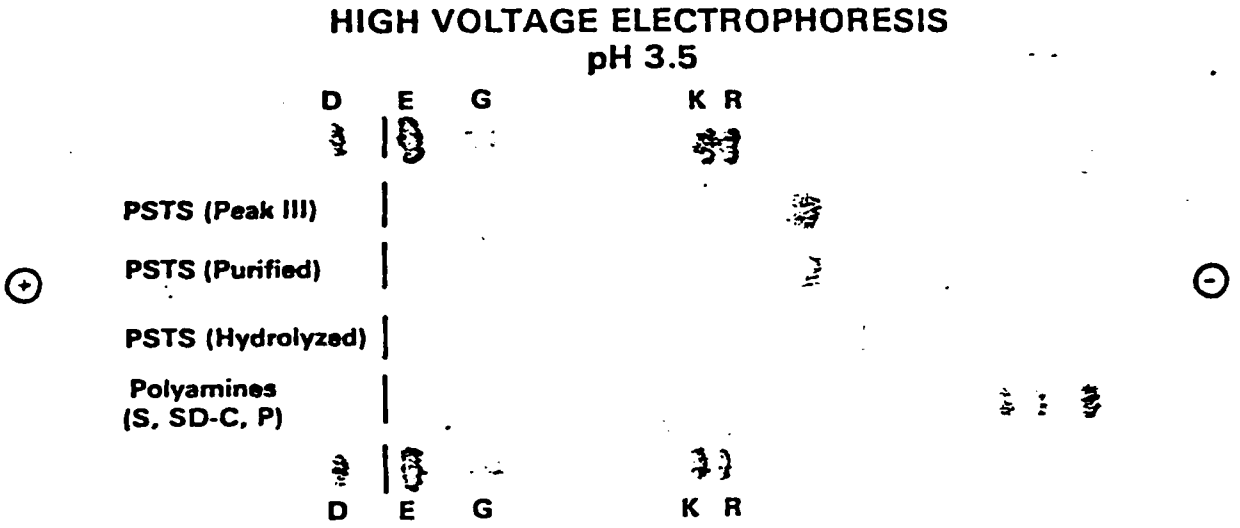


Figure 2. High-voltage electrophoresis of Fraction 11 derived from the pooled serum of patients who became anergic after major cardiovascular surgery before and after purification by repeat high-voltage electrophoresis. Purified Fraction 11 was then subjected to acid hydrolysis. This yielded several amino acids and the basic ninhydrin positive molecule shown. This basic molecule, however, is not as basic as the known polyamines. Reference amino acids are shown in the top and bottom lines. R = lysine.

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Correlation Between Anergy and a Circulating Immunosuppressive Factor Following Major Surgical Trauma

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In order to clarify the relationship between anergy and immunosuppressive activity in the serum, we studied 46 previously well patients before and at three, five, seven and 28 days after surgery. Delayed hypersensitivity was measured by skin testing with four common recall antigens, and serum immunosuppressive activity was determined by the ability of the patient's serum in 10% concentration to suppress by 50% or more the phytohemagglutinin (PHA) stimulation of normal human lymphocytes as compared to pooled normal serum. Prior to surgery, all patients manifested delayed hypersensitivity to one or more antigens, and no patient had immunosuppressive serum. Fifteen patients underwent minor surgery under general anesthesia and did not develop anergy or immunosuppressive serum. Thirty-one patients underwent major cardiovascular surgery. Thirteen of these patients became anergic by day 3 after operation, and 11 of the 13 developed immunosuppressive serum. Eighteen patients maintained delayed hypersensitivity after major surgery, and only three developed immunosuppressive serum. The correlation between anergy and immunosuppressive serum was highly significant ($p < 0.001$). There was a significant difference in the degree of suppressive activity in the serum of the anergic and reactive patient groups for each postoperative day studied until day 28, when there was recovery of delayed hypersensitivity and lack of immunosuppressive serum. The occurrence of postoperative anergy and immunosuppressive serum was not related to the patient's age, sex, number of perioperative blood transfusions or duration of anesthesia but was associated with an increase in postoperative infectious complications ($p < 0.05$) and in postoperative days in the hospital ($p < 0.01$). Pooled immunosuppressive serum from anergic patients was fractionated by ion exchange chromatography, gel filtration and preparative high voltage electrophoresis. The majority of the immunosuppressive activity could be accounted for by an electrophoretically homogenous polypeptide-containing fraction not identified in the serum of patients undergoing minor surgery or in normal individuals. We conclude that anergy occurring after major operative trauma is associated with the appearance of a circulating immunosuppressive molecular species and that these events are in turn associated with increased patient morbidity and increased length of hospitalization.

ANERGY IS FOUND in surgical patients with nutritional deprivation or advanced malignancy and is associated with an increased incidence of sepsis and mortality.[3, 6-8] Since restoration of delayed hypersensitivity responsiveness has been reported in such patients following parenteral hyperalimentation,[3,9] this suggests an underlying mechanism for the anergy observed in these depleted individuals. On the other hand, there are conflicting reports as to whether or not major surgical trauma in nondepleted patients is followed by anergy.[5,10] Moreover, if anergy does occur under these circumstances, it is not clear what the mechanism is and what effect, if any, the anergic state has on patient morbidity and mortality. A good deal of recent investigative work has focused on defects in polymorphonuclear leukocyte function detected in anergic surgical patients.[1,4,6] While polymorphonuclear leukocytes clearly play an important role in the defense against bacterial infection in man, they have not been shown to be obligatory participants in delayed hypersensitivity responses.[11] These responses are mediated by specifically sensitized T lymphocytes, which in turn elicit the nonspecific cooperation of macrophages.

We have recently reported that major operative trauma is often followed by the appearance in the serum of a circulating factor or factors suppressive of T-lymphocyte activation.[2] We therefore undertook the present investigation in a group of well-nourished

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TABLE 1. *Skin Test, Delayed Hypersensitivity Response to Four Recall Antigens (Mumps, SK-SD, PPD, Candida)*

	Grade	Response (Diameter of Induration)
Anergic	0	0
	1	< 5 mm for 1
Reactive	2	≥5 mm for 1 or ≥3 mm for 2
	3	≥10 mm for 1 or ≥10 mm for 1 and ≥5 mm for another
	4	20 mm for 1 or ≥15 for 1 with 10 mm for another or ≥10 mm for 2 or more

surgical patients, none of whom had malignancy, to determine whether or not the appearance of circulating immunosuppressive factors in the serum postoperatively was associated with the manifestation of anergy and whether the anergic state was in turn associated with an altered patient prognosis. We were also concerned with the purification and characterization of the immunosuppressive substance or substances detected in the serum of these surgically traumatized patients.

Patient Population and Methods

Forty-six patients were studied. Fifteen of these patients received general anesthesia for minor surgical operations. Seven underwent inguinal hernia repair, two had dilatation and curettage, three had multiple dental extractions, and three had orthopedic manipulations. The age of this patient population ranged from 35 to 75 years, with a mean age of 56 years. There were 13 males and two females.

Thirty-one patients underwent major cardiovascular surgery under general anesthesia. Sixteen of these patients had abdominal aortic aneurysm resections. Ten underwent coronary artery bypass grafts, and five underwent aortic or mitral valve replacement, with or without coronary artery bypass grafting. The age of this patient group ranged from 42 to 75 years. The mean age was 62 years. There were 27 males and four females. No patient judged clinically to be nutritionally depleted was included in this study, and no patient had cancer. In addition, nine healthy normal volunteers, ranging in age from 24 to 63 years, were used as control serum donors in some of the studies. Informed consent was obtained from all patients before studies were initiated.

All patients were skin tested with four recall antigens for delayed hypersensitivity responsiveness 2 days prior to surgery. The antigens were mumps skin test antigen (Eli Lilly & Co., Indianapolis, IN), 0.1 ml; 50 units of streptokinase-streptodornase (SK-SD) (Lederle Laboratories, Pearl River, NY); intermediate strength tuberculin purified protein derivative (PPD) (Merck, Sharp and Dohme, West Point, PA), 0.1 ml; and *Candida* skin test antigen (Greer Laboratories, New York, NY), 0.1 ml. All skin tests were read at 24 and 48 hours and were scored according to the system listed in Table 1. Responses were graded by the diameter of the area of induration. Patients were considered to be anergic if they had responses of grades 0 and 1 and reactive if they had responses of grades 2, 3 and 4. No patient who was anergic preoperatively was included in these investigations. Skin tests were repeated in all patients on the second postoperative day, the seventh postoperative day and the twenty-eighth postoperative day.

Histamine (Eli Lilly), 0.5 mg, was injected intradermally as a control for an intact inflammatory response in the postoperative period. Thirty-milliliter venous blood samples were drawn beginning 2 days preoperatively and then on the third postoperative day, the fifth postoperative day, the seventh postoperative day, the fourteenth postoperative day (in some patients) and on the twenty-eighth postoperative day. In order to obtain serum samples, the blood was allowed to clot and retract, and the serum was removed by centrifugation at 2000 × g for 30 min and stored in the cold.

In Vitro *Assay of Immunosuppressive Activity*

Serum samples were tested for immunosuppressive activity *in vitro* by studying their ability to inhibit phytohemagglutinin (PHA)-induced normal human lymphocyte proliferation. Heparinized venous blood was obtained from the normal donors, and after gravity sedimentation of the erythrocytes for 2 hours at 20°, the serum layer was placed on sterile nylon wool columns and eluted with Eagle's minimal essential medium (MEM). After washing in MEM, the cells were counted and tested for viability by trypan blue dye exclusion. The procedure yielded a preparation of small lymphocytes, 95% or more pure and 95% or more viable. The micro method was used for testing lymphocyte stimulation. In the wells of Microtest® plates (Falcon Plastics) 2.5×10^5 lymphocytes were placed in 0.2 ml of MEM containing 1% glutamine, 5% fetal calf serum, 100 units of penicillin and 100 μg of streptomycin per milliliter and a range of stimulatory doses of purified PHA (2.5, 5 and 10 μg/ml). Serums to be tested for immunosuppressive activity were added to the culture medium in 10% concentration. Controls included cultures with no additions and those with 10% pooled normal serum. The same normal serum pool was used for all experiments. Microtest plates were then in-

cubated in a 5% CO_2 water-saturated environment at 37° for 48 hours. ^{3}H-thymidine, 1 μCi, was then added to each well. The cultures were processed 16–18 hours later by a Mash II® microharvester (Microbiological Associates) and counted in a Packard liquid scintillation counter. All determinations were performed in triplicate. Some wells in each experiment were used for a trypan blue viability determination of the cells incubated with or without the serums being tested. No cytotoxic serums were found in these experiments. Immunosuppression *in vitro* was calculated by the formula

$$\% \text{ Suppression} = 1 - \frac{\text{CPM, experimental wells with PHA} - \text{CPM of control wells without PHA}}{\text{CPM of control wells with PHA} - \text{CPM of control wells without PHA}} \cdot 100$$

In these studies, suppression of PHA stimulation by experimental serum of 50% or more when compared with control serum was considered significant.

Isolation of Suppressive Material from the Serum

Ten-milliliter samples of serum were fractionated by diethylaminoethyl (DEAE) cellulose ion exchange chromatography in 0.005 M acetate buffer (pH 5.0). The protein peaks from this separation were then recovered by lyophilization and tested for immunosuppressive activity in tissue culture as described above. Lyophilized protein, 100 mg, was then dissolved in distilled water and placed on a G-25 Sephadex® column. The protein peaks from this column were then similarly recovered by lyophilization. After testing for immunosuppressive activity *in vitro*, the G-25 fractions were dissolved in distilled water and acetic acid, pH 3.5, and placed in 10–20-mg aliquots on paper strips for high voltage electrophoresis along with reference amino acids. A portion of the electrophoresis strip was stained with ninhydrin, and the remainder of the strip was cut into fractions containing the various ninhydrin staining moieties. The fractions were then eluted from the paper with distilled water-acetic acid solution and recovered by lyophilization. Each of the recovered, presumably peptide-containing moieties was then tested for suppressive activity in tissue culture.

In Vivo *Assay of Immunosuppressive Activity*

To confirm the results of the *in vitro* tissue culture assay, suppressive and nonsuppressive fractions obtained by G-25 gel filtration were tested for immunosuppressive activity *in vivo* in mice by the Jerne hemolytic plaque assay. Adult C3H mice (Jackson Laboratories) were injected intraperitoneally with test or control fractions 24 hours before the intraperitoneal injection of 4×10^8 sheep erythrocytes. Four days later their spleens were harvested, and the numbers of plaque-forming cells were enumerated as described previously.[7] Duplicate determinations were performed in each of five animals per experimental group.

Cortisol Determinations

Serum cortisol determinations were performed in the hospital laboratory by the competitive protein-binding method.

Results

Correlation of Anergy with Suppressive Activity in the Serum

Of the 15 patients undergoing minor surgical procedures under general anesthesia, no patient became anergic at any time in the postoperative period and no patient developed immunosuppressive serum as determined by the ability of the serum in 10% concentration to suppress by 50% or more the PHA stimulation of normal human lymphocytes (Table 2). However, of the 31 patients who underwent major cardiovascular surgery, 13 became anergic to the skin test antigens by postoperative day 3, and of these 13 patients, 11 developed immunosuppressive serum. Of the 18 patients who remained reactive to the skin test antigens, only three developed immunosuppressive serum at any time in the postoperative period. By χ^2 analysis the correlation between immunosuppressive serum and anergy in the patients undergoing major cardiovascular surgery is highly significant ($p < 0.001$).

As shown in Figure 1, the suppressive activity in the serum of the entire group of patients who became anergic in the postoperative period was significantly

TABLE 2. *Correlation of Anergy with Suppressive Serum Three Days After Surgical Trauma*

Surgery	Skin Test	Immunosuppressive Serum*	Nonsuppressive Serum	Total No
Minor	Positive	0	15	15
	Negative	0	0	0
Major	Positive	3	15	18
	Negative	11	2	13

* Immunosuppressive serum at 10% concentration was more than 50% suppressive of PHA stimulation of normal human lymphocytes
† Chi square with Yate's correction. ‡ $p < 0.001$

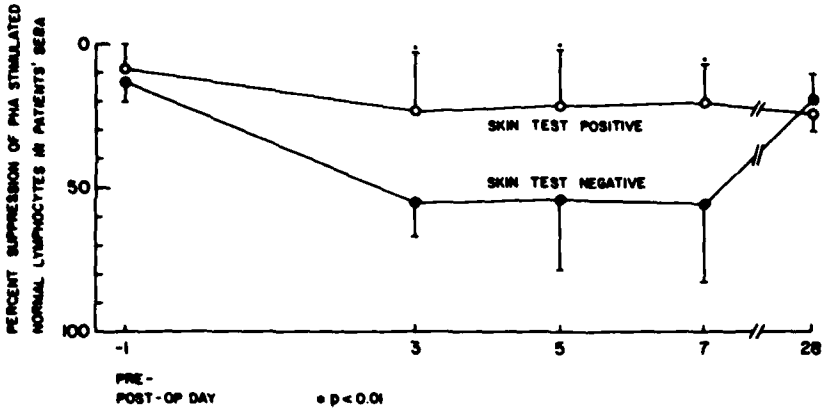

FIG. 1. The immunosuppressive activity in the serum of negative skin test and positive skin test patients following major cardiovascular surgery in the present study. Results are presented as mean percentage suppression of PHA stimulation of normal peripheral blood lymphocytes (+ S.D.) by test serum in 10% concentration. The two groups differ significantly on postoperative days 3, 5 and 7 as determined by the paired *t*-test ($p < 0.001$).

greater than that in the serum of patients who remained reactive to the skin test antigens for the first 7 days postoperatively. By day 28 when the patients were seen as outpatients, significant suppressive activity in the serum had disappeared. By day 28 the patients had also regained responsiveness to the skin test antigens. All patients responded normally to intradermal histamine in the early postoperative period. Serum cortisol levels were within the normal range in pooled and selected individual postoperative samples.

Association of Anergy and Immunosuppressive Serum with Patient Morbidity

As noted in Table 3, 11 of the patients undergoing major surgery developed both anergy and immunosuppressive serum in the postoperative period, while 15 manifested neither. The two groups did not differ significantly from one another in age or sex. They also did not differ from one another with respect to the number of perioperative blood transfusions received or the duration of anesthesia. However, the patients with anergy and suppressive serum developed significantly more infectious complications that required antibiotic therapy in the postoperative period. These were predominantly pulmonary and urinary tract infections. Also the group of patients with anergy and suppressive serum spent significantly more days in the hospital postoperatively.

Isolation of Suppressive Material from the Serum

Pooled immunosuppressive serum from eight of the patients undergoing major surgery who developed anergy and suppressive serum in the postoperative period was subjected to DEAE cellulose ion exchange chromatography. The peaks obtained were tested for immunosuppressive activity at 5, 2 and 1 mg/ml. Controls included pooled serum from eight patients who underwent minor surgical procedures and pooled serum from eight normal individuals. Suppressive activity from the immunosuppressive serum from patients in the major surgical group was found principally in the first two peaks from the DEAE column.

These suppressive peaks from DEAE chromatography were then subjected to gel filtration on a G-25

TABLE 3. *Major Surgical Patients*

Anergy and Suppressive Serum	Blood Transfusions	Duration of Anesthesia (hr)	Postop. Infectious Complications	Postop. Days in Hospital
Yes n = 11, M = 10, F = 1, av. age = 63	2.33 ± 0.89*	4.02 ± 0.31*	6/11 $p < 0.05$	13.5 ± 4.2* $p < 0.01$
No n = 15, M = 13, F = 2, av. age = 62	2.33 ± 0.87	4.11 ± 0.24	1/15	8.8 ± 2.2

* Mean ± S.D.

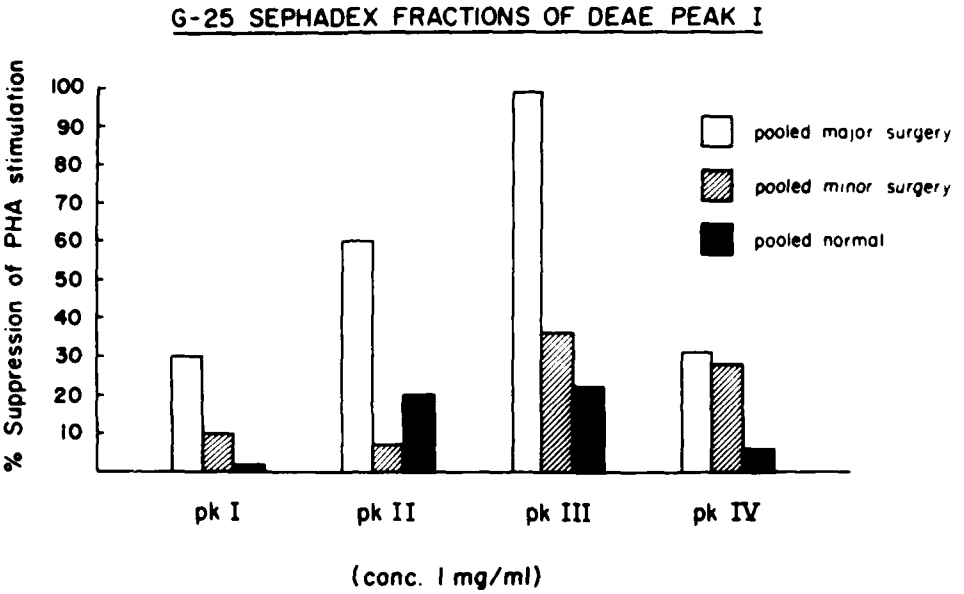

FIG. 2. Suppressive activity of fractions of serum obtained by G-25 Sephadex chromatography and tested at 1 mg/ml concentration. Results are presented as mean percentage suppression of PHA stimulation of normal human lymphocytes. It is apparent that the suppressive activity in the serum of patients following major cardiovascular surgery is found chiefly in peak 3. Pooled serum from patients undergoing minor surgery or from normal individuals contains little suppressive activity.

Sephadex column, and the resultant polypeptide peaks were lyophilized and tested for immunosuppressive activity in tissue culture at concentrations of 2, 1 and 0.5 mg/ml. As noted in Figure 2, the immunosuppressive activity in the serum from suppressed major surgical patients was located principally in peak 3 from the G-25 column. Very little suppressive activity was recovered from serum from patients subjected to minor surgery or from normal individuals.

G-25 peak 3 from suppressed patients undergoing major surgery was also tested for immunosuppressive activity in the mouse. It is apparent from Table 4 that G-25 peak 3 at a dose of 5 mg per mouse produced very significant suppression of the plaque-forming cell response to sheep erythrocytes in contrast to G-25 peak 1. At this dosage the concentration of G-25 peak 3 in mouse serum was calculated to be approximately the same as in the serum of suppressed major surgical patients.

Finally, G-25 peak 3 from suppressed patients undergoing major surgery was further fractionated by preparative high voltage electrophoresis. It was found that the immunosuppressive activity was recovered principally in a highly basic fraction, fraction 11 (Table 5). Fraction 11 was not detectable in the serum of patients who had undergone minor surgery or in the serum of normal individuals. The same area in the electrophoresis strip contained negligible immunosuppressive activity in these control groups.

Fraction 11 from suppressed major surgical patients was subjected to acid hydrolysis and yielded amino acids plus a so far unidentified ninhydrin staining basic component, more basic than known amino acids but less basic than known polyamines.

Discussion

These results clearly demonstrate that following major cardiovascular surgery, temporary anergy fre-

TABLE 4. *Effect of Major Surgery Serum Fractions on Direct Plaque-Forming Cell Response to SRBC in C3H Mice*

Treatment	Plaques per 10^6 Spleen Cells (±S.D.)	Per Cent Suppression
None	485 ± 7.7	
G-25 peak 1		
5 mg	502 ± 22.4	0
1 mg	464 ± 6.1	4
G-25 peak 3		
5 mg	212 ± 12.1	57
1 mg	466 ± 15.6	4

TABLE 5. *Serum Fractionation: High Voltage Electrophoresis of G-25 Sephadex Peak 3 (% Suppression of PHA Stimulation at 1–2 μg/ml)*

	Fraction											
	(+)	1	2	3	4	5	6	7	8	9	10	11 (−)
Major surgery		14	12		24		17	21	0	24		63
Minor surgery		0	13	0		5	12	11	13	3	0	10
Normal		0	0	9	0	5	35	13	7	8	37	0

quently appears in apparently well-nourished patients who do not have malignancy. Our results do not agree entirely with those reported by Slade et al.,[10] who found that patients undergoing major surgery consistently show decreased delayed hypersensitivity responses postoperatively, and our findings are also clearly at variance with those of Pietsch et al.[5] who concluded that skin test responses were not altered by major surgery in apparently nondepleted patients. The reasons for these discrepancies are not entirely clear, but the magnitude of the operative trauma may well be related to the incidence of postoperative anergy in a well-nourished patient population.

Anergy in the patients in the present study was accompanied by the appearance of circulating immunosuppressive activity in the serum, which blocked the activation of T lymphocytes from normal individuals but was not cytotoxic to these cells. The appearance of anergy and immunosuppressive activity in the serum was not the direct result of general anesthesia, since a group of 15 patients undergoing minor surgery under general anesthesia developed neither anergy nor immunosuppressive serum in the postoperative period. Among the patients undergoing major cardiovascular surgery, those who developed anergy and immunosuppressive serum could not be distinguished from those who did not on the basis of age, sex, number of perioperative blood transfusions or duration of anesthesia. However, patients with anergy and immunosuppressive serum had significantly more infectious complications requiring antibiotic therapy in the postoperative period than those patients who remained responsive to recall antigens and did not develop immunosuppressive serum. While there were no postoperative deaths, patients with anergy and immunosuppressive serum also spent significantly more days in the hospital postoperatively than patients who remained responsive.

The mechanism underlying the appearance of anergy and immunosuppressive serum in some of the patients undergoing major cardiovascular surgery in the present study remains obscure. Anergy in these patients cannot be explained on the basis of a generalized incapacity to mount an inflammatory response, since all patients responded normally to intradermal histamine. None of the patients was apparently nutritionally depleted, and no patient had a known malignancy. We have previously demonstrated that immunosuppressive serum in surgical patients is not related to serum cortisol concentrations,[2] and serum cortisol determinations in the patients reported here showed normal levels postoperatively. The most obvious explanation for the present observations appears to be that major operative trauma in itself triggers a temporary inhibition of cellular immunity, possibly mediated by circulating immunosuppressive factors.

The present results also shed some light on the nature of the immunosuppressive activity in the serum of the patients who became anergic after major surgery. In these individuals the majority but not all of the immunosuppressive activity can be accounted for by a polypeptide-like fraction which from its behavior on gel filtration has a molecular weight of approximately 1000 daltons. This material can be recovered as a homogeneous molecular species by high voltage electrophoresis. The highly basic character of this material on electrophoresis makes it unlikely that it is a conventional polypeptide; however, it is not as basic as any of the known polyamines. This material was not recoverable in detectable quantities by identical fractionation of the serum from normal individuals or from patients who had undergone minor surgical procedures.

It is tempting to speculate that the appearance of an immunosuppressive molecular species in the serum of patients following major surgical trauma may be the cause of the anergy seen in these individuals, since there is a statistically significant association between these phenomena. The fact that the gel filtration fraction in which the patients' serum suppressive activity was concentrated also suppressed the ability of mice to mount an antibody response to a T-cell dependent antigen adds weight to this hypothesis. However, a causal relationship between this suppressive substance and clinical anergy cannot yet be claimed to be established.

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Discussion

DR. GEORGE H. A. CROWLEY, JR. (Boston, Massachusetts): Specifically in relation to John Mannick's paper, the more I look at trauma and sepsis, the more I realize that they have the same effects metabolically, and in a variety of other ways physiologically. That is, if we assume that the trauma patient has gone beyond the shock phase. The response is very similar then to that of the septic patient.

For some time we have been interested in circulating factors of the small nonprotein peptide type that Dr. Mannick has discussed. (slide) This is a thin-layer chromatograph with a ninhydrin stain that shows in that fraction around 3000–5000 molecular weight a great difference between normal plasma and septic plasma.

Here is what is found when that same fraction from 1000 to 10,000 molecular weight is examined by column chromatography. The point is that the 206 nanometer light exposure, which activates the peptide bonds, demonstrates two large peaks in the fraction from a septic or traumatized patient that are just barely discernible in the plasma fraction from a normal person. This demonstrates that there are many peptide substances circulating under these conditions that are not present in the normal individual.

(slide) It is possible to show a remarkable correlation of the presence of these substances with the clinical state. If one binds these peptides to Sephadex, it is possible to make an antibody. We see here reactions in a series of septic patients, which are absent in the normal person when this immunologic diffusion test is done.

(slide) What is the significance of this? This slide emphasizes the importance of how these agents affect the metabolism of incubated muscle cells. We see virtually no change in the metabolism when saline solution is added. There is a response to insulin in terms of CO_2 production. If normal human plasma or normal plasma fraction are added we get the same response, but if we add the septic plasma, or the septic fraction, we get a suppression of insulin response. That is just one metabolic phenomenon. I can tell you from experience that the same thing happens for protein synthesis and a variety of other parameters which we have measured.

What I'm really saying is that, to me, the bottom line of all this work, and the important response in which we are interested is protein synthesis. After all, T-cell function depends on its ability to make a protein pretty quickly, and I would say that this probably is the common denominator in all of these reactions. The same agent that Dr. Mannick has so elegantly demonstrated to you this morning as well as many other peptides are probably at work in the other phenomena the other two speakers described this morning.

DR. JONATHAN L. MEAKINS (Montreal, Quebec): Dr. Mannick's paper is a very exciting one, and approaches the problems of immune regulation in a normal, well-nourished population. We have approached this from a slightly different point of view, in terms of decreased host resistance to infection and immunoregulation.

(slide) Dr. Christou recently presented this information on the effect of anergic patients' serum on neutrophil and lymphocyte chemotaxis. The test cells are normal, and it is apparent that relatively anergic and anergic serum reduces PMN chemotaxis and lymphocyte chemotaxis to the anergic range.

(slide) Trauma patients, studied in the emergency department as they are admitted to the hospital, are seen to have abnormal chemotaxis 2–12 hours after injury. Anergy subsequently developed in all of them. This abnormality of their chemotaxis must surely be mediated by a serum factor to appear so promptly.

(slide) We have looked at the concentration of anergic serums required to inhibit chemotaxis and find that there are two inflection points of inhibition of PMN chemotaxis, one at 10% serum and a second one at about 50%. This is a highly reproducible curve, even though the second point of inflection does not appear to be great.

So my first question would be whether or not there are other inhibitors in Dr. Mannick's serum which might correspond to these findings.

(slide) Utilizing the concept that there were two inhibitors, we looked at G-200 Sephadex chromatography and found that there are again two inhibitors of chemotaxis. Data are confirmed using sucrose density gradients, as well as isoelectric focusing. These inhibitors are about 360,000 and 120,000 molecular weight.

More recently, we found smaller inhibitors, and it leads to my basic question. I wonder if Dr. Mannick could comment upon the nature of these multiple inhibitors, and whether they are all part of a common, or similar, immunoregulatory system.

DR. DONALD L. MORTON (Los Angeles, California): Dr. Jack Roth and I have done some studies with conclusions somewhat similar to those of Dr. McLoughlin and his colleagues.

We looked at the effect of surgical trauma on immunosuppression in patients with cancer, but our data were organized in a slightly different way. We compared patients with minor trauma, such as that from regional lymphadenectomy, with patients whose operative procedures invaded the thoracic or abdominal cavities. We found no correlation with the length of operation but did find that, if the abdominal or thoracic cavities were entered, the patients were more immunosuppressed. Also, if tumor was completely resected, immunosuppressive factors disappeared even in patients who were anergic preoperatively and who had major surgical trauma.

In our series there was a correlation between blood transfusion and degree and duration of immunosuppression. The most immunosuppressed patients were those undergoing cardiopulmonary bypass.

The duration of immunosuppression in our series was similar. One patient was immunosuppressed for six weeks, but usually the patient's immunocompetence returned in seven to ten days.

Finally, I would like to ask Dr. Mannick if there was a difference in the degree of immunosuppression between patients who had cardiopulmonary bypass and those who had major aortic resections.

DR. STANLEY M. LEVENSON (Bronx, New York): I wonder if Dr. Mannick can tell us something about the specific amino acid composition of the active fraction or fractions. I am interested in that particularly because in the late '40's and early '50's my colleagues and I described an amino conjugate fraction which appeared in the serum of previously healthy animals and previously healthy men who were injured. The concentration of this fraction correlated with the severity of the injury. It is a dialyzable compound, or group of compounds, and increased remarkably in patients with renal dysfunction.

I was wondering whether there may be some similarity between the active fraction or fractions Dr. Mannick and his colleagues have isolated and the amino conjugate fraction we worked with in terms

of amino acid composition. I would also like to ask Dr. Mannick if he has looked at patients with renal dysfunction following injury, to see whether there is a still higher increase in the fraction or fractions he is looking at. If so, this may be one of the reasons why a patient with renal failure is particularly susceptible to infection.

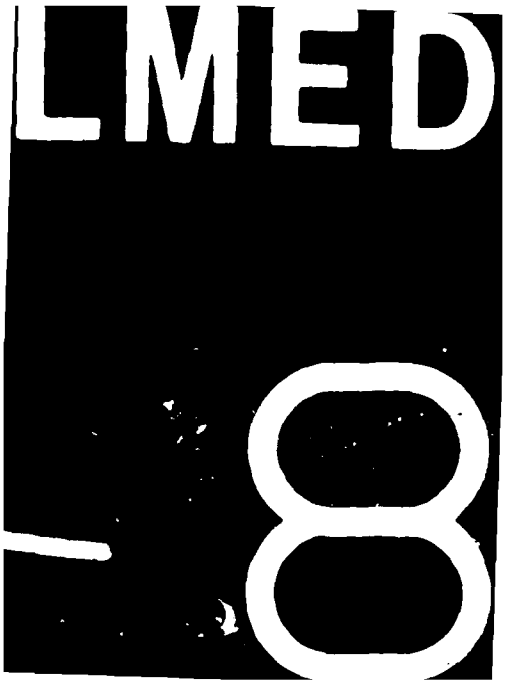
DR. JOHN A. MANNICK (Closing discussion): In reply to Dr. Clowes, I do believe that we are now finding some functions for that myriad of polypeptide molecules that circulate around in everyone's serum, whose function has heretofore been unknown, and I suppose that they represent a few words in the biochemical language that cells use to communicate with one another, and that language has by no means been translated yet.

I don't know what relationship the factors that Dr. Clowes has been working with have to the one we have been talking about. I am not sure whether our factor has any metabolic effect on lymphocytes that is other than transient, simply because cells that do not rosette very well from traumatized patients in our laboratory upon washing multiple times will then rosette quite actively, and the wash material does contain the sort of molecule we have been talking about.

In answer to Dr. Meakins, I think that his idea is an intriguing one, namely, that the immunoregulatory system may have some features similar to the complement system; for example, breakdown products of one activity may subsume other activities, and we may be talking about pieces of molecules that once did something else, and now affect a different cell type. I think that is perfectly possible, but I don't know anything else to say about it at this time, other than to admit the possibility.

In reply to Dr. Morton, cardiopulmonary bypass patients and aneurysm resection patients, which were the two groups we were looking at, really behaved the same in terms of percentage that developed anergy and the percentage that had suppressive serum. So in this instance, surprisingly enough, cardiopulmonary bypass did not seem to be any different than aneurysm resection.

In answer to Dr. Levenson, I have been intrigued by that early paper of his, and he may be right. He may, in fact, have identified this material. I just don't know whether they are similar or not. The amino acid composition of our material I don't think I can give him with any confidence. We do have a sample of this material in the hands of the Molecular Biology Institute at Hoffman-La Roche, and we hope to have some information about its true nature in a few weeks.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