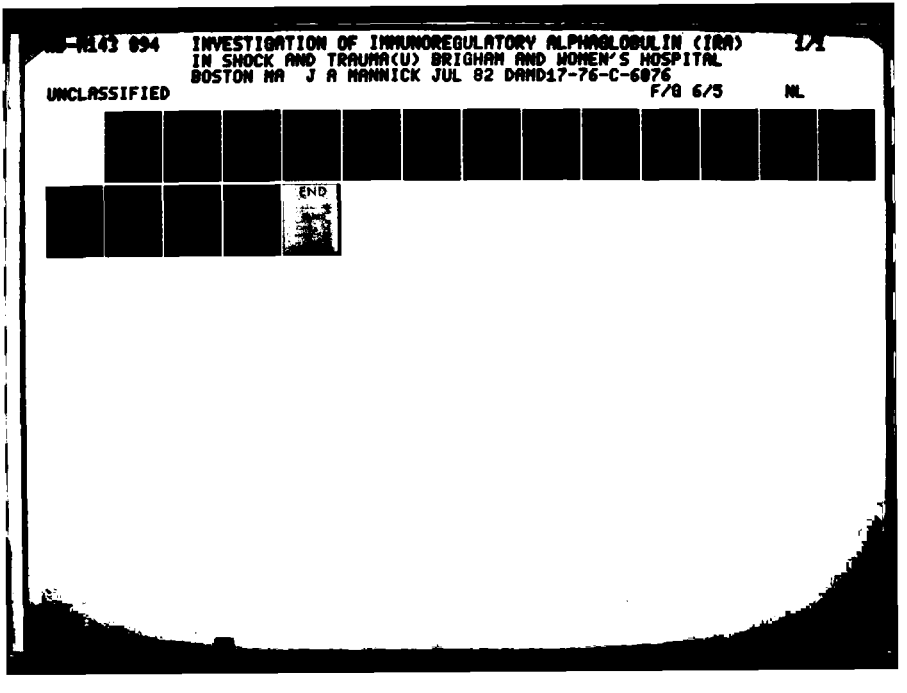
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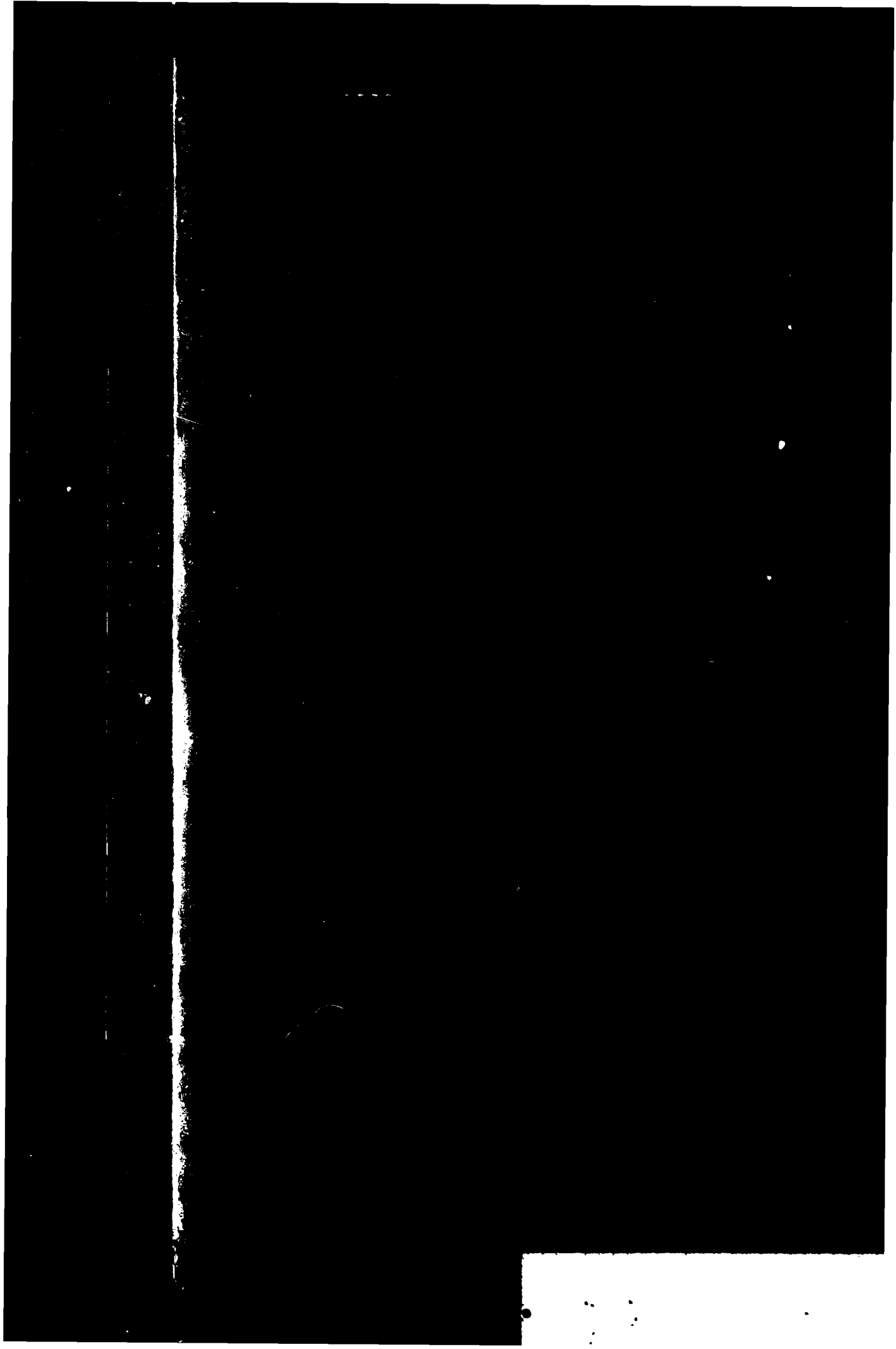

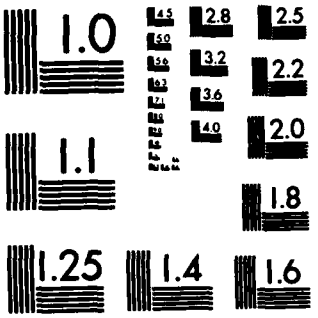

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Investigation of Immunoregulatory Alphaglobulin (IRA) in Shock and Trauma

Annual Report

John A. Mannick, M.D.

July 1982

Supported By

US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Brigham and Women's Hospital
Boston, Massachusetts 02115

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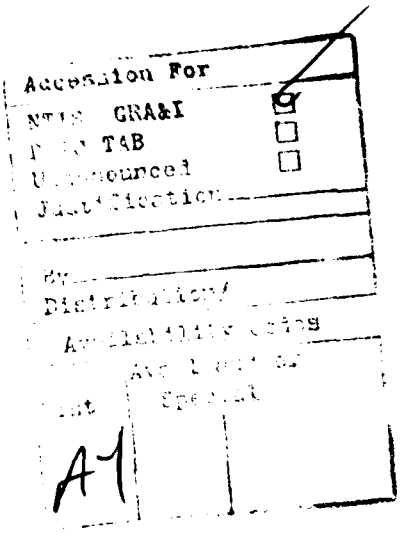
FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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



PROGRESS REPORT

During the final year of the project burn patients have been a major focus of our clinical studies. Burn patients with greater than 30% body surface area burn were skin tested for hypersensitivity responsiveness to 4 standard recall antigens and were sensitized to dinitrochlorobenzene (DNCB). The results of the skin tests were compared on each occasion with the ability of the patient's serum in 10% concentration to suppress normal human lymphocyte stimulation by phytohemagglutinin (PHA) in tissue culture. Seventy-eight serum samples were taken and the immunosuppressive activity related to the presence or absence of coexistant cutaneous anergy ~~(see Figure 1 in the appendix)~~. Cutaneous anergy or relative anergy was present on 40 occasions and 25 (63%) of the associated serum samples at 10% concentration suppressed normal lymphocyte blastogenesis with PHA by more than 50%. There was a normal delayed hypersensitivity reaction on 38 occasions and this was associated with suppressive serum on 11 occasions (28%). By X^2 analysis there was a correlation between anergy and coexistant serum immunosuppression of greater than 50% ($X^2 = 8.71$, p 0.005).

The 78 serum samples represented serial measurements in 21 patients. Nine of the 10 (90%) patients that developed anergy also developed suppressive serum at some stage of their illness whereas only 14 of 11 (36%) who did not become anergic developed suppressive serum. The mean greatest immunosuppression in the reactive group was 27.7% which is also a significant difference (p 0.01 using Student's t test). The relationship of anergy to serum immunosuppression in two patients over the course of their illness is shown in Figure 2 in the appendix. Serum suppressive activity in burn patients did not correlate with plasma levels of PGE_2, as described by radio-immunassay, with plasma cortisol levels, or levels of circulating endotoxin.

On 33 occasions skin testing and determination of the patient's peripheral blood lymphocyte response to PHA were done synchronously. (see Figure 1 in appendix) On 20 of these occasions anergy or relative anergy was present. A greater than 50% impairment of the peripheral blood lymphocyte PHA response was related to anergy or relative anergy on 15 (75%) of these occasions. ON 13 occasions the skin test was reactive and this was associated with lymphocyte suppression in 3 cases (23%). This difference was statistically significant ($X^2 = 7.35$, p 0.01).

Anergy did not correlate with predicted survival in this population of burn patients but was a good predictor of the actual survival of these individuals. This work has been published in the Annals of Surgery.

Most burn patients did not demonstrate an impaired response of their washed peripheral blood lymphocytes to PHA stimulation in vitro. However, a depressed PHA response was associated with severe infection and high mortality. Four of the 7 patients who manifested this finding died. Conversely, only one burn patient died without manifesting severe depression of the peripheral blood lymphocyte response to PHA stimulation in vitro and this patient died of pulmonary embolism. The presence of circulating leukocytes suppressive of the response of lymphocytes from normal donors to PHA was studied serially in 7 burn patients (27 samples) and correlated significantly with depression of PHA-induced blastogenesis ($r=0.72$, p 0.01) in these patients. These studies suggest that the presence of a circulating immunosuppressive factor(s) is a very common consequence of 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 the response of peripheral blood lymphocytes to PHA as compared with simultaneously studied normal controls and the appearance of circulating

suppressor leukocytes is associated with a grave prognosis. This work has been published in the Surgical Forum and in the Annals of Surgery.

During the past year we have also begun to study a series of patients who have sustained major accidental trauma (injury severity score (ISS) 20-40) and while these studies are not yet complete they show that major trauma patients resemble burn patients in that suppressive serum is a ubiquitous finding in these individuals and that impending septic death is associated with diminished lymphocyte PHA response.

Investigations of the biological effects of low molecular weight serum suppressive material (Peak 3 or 4 from Sephadex G-25 chromatography) from trauma and burn patients have continued in an animal system. Low molecular weight material from burn patients, from normal individuals and from patients following major trauma has been injected into A/Jax mice which were then challenged with and LD 20 dose of Listeria monocytogenes organisms, usually 1×10^5 organisms. Listeria was selected because in common with a number of gram negative bacteria it is known to require an intact cellular immune response for its elimination. Low molecular weight material from burn patients and from trauma patients at a dose of 5 mg per mouse induced 60-100% mortality. Control mice injected with higher molecular weight material from the same burn and trauma serum did not manifest increased mortality and the low molecular weight material administered without Listeria did not induce mortality. We believe that these experiments offer convincing evidence that the circulating low molecular weight suppressive material from burn and trauma patients suppresses host resistance to some microorganisms. This work will be published in the Archives of Surgery (see Table 1 in appendix).

During the past year we have also initiated experiments to determine the effect of low molecular weight material (Sephadex Peak 3 or 4) from trauma

and burn patients on the induction of suppressor cells in mice. Preliminary results have shown that the spleens of mice injected with this low molecular weight material are able to suppress the PHA response of normal syngeneic mouse spleen cells in a graded dose response fashion. (see Table II and Figure 3 in the appendix)

During the past year we have also pursued the purification of the active fraction(s) present in immunosuppressive serum from trauma and burn patients. Pools of serum from individuals who have undergone major surgical trauma or have suffered major burns or accidental trauma have been subjected to DEAE cellulose chromatography and the initial two peaks were found to be active. These peaks were further fractionated by gel filtration on Sephadex G-25 columns. The low molecular weight fraction estimated 1000-5000 daltons consistently eluted at 2/3 column volume and was called Peak 3 or 4 depending upon the number of peptide peaks in the sample. This fraction was found to contain a majority of the suppressive activity as determined by its ability to suppress PHA stimulation or normal human lymphocytes and tissue culture without cytotoxicity. We found that the suppressive moiety(s) in the active G-25 peak could not be resolved by ion exchange chromatography or further gel filtration and was of too low molecular weight to be isolated by isoelectric focusing or polyacrylamide gel electrophoresis. Therefore, the active G-25 peak was further fractionated by preparative high-voltage paper electrophoresis at pH 3.5. Individual ninhydrin positive moieties were eluted from the electrophortogram and recovered by lyophilization. These fractions were tested for suppressive activity <u>in vitro</u> and the majority of the activity appeared to be in a highly basic fraction as noted in the previous progress report. This highly basic molecular species has not been recovered in detectable amounts from similarly processed samples of serum from patients who have suffered minor trauma or from normal volunteers. This basic fraction has also been shown to inhibit antibody formation in the

Mishell-Dutton system in vitro and to inhibit the generation of cytotoxic cells in mixed lymphocyte culture (see Figure 4 and Table III in the appendix). It is clear, however, that further work will be necessary before the molecular species responsible for the suppressive activity from peaks 3 or 4 from Sephadex G-25 chromatography can be characterized.

APPENDIX

LEGEND

Figure 1: Shows the association of serum suppressive of lymphocyte blastogenesis and impaired blastogenesis of peripheral blood lymphocytes with anergy in 21 burn patients on 78 occasions. It is apparent that suppressive serum and impaired lymphocyte blastogenesis were found much more frequently in anergic patients than in patients with normal delayed hypersensitivity responses.

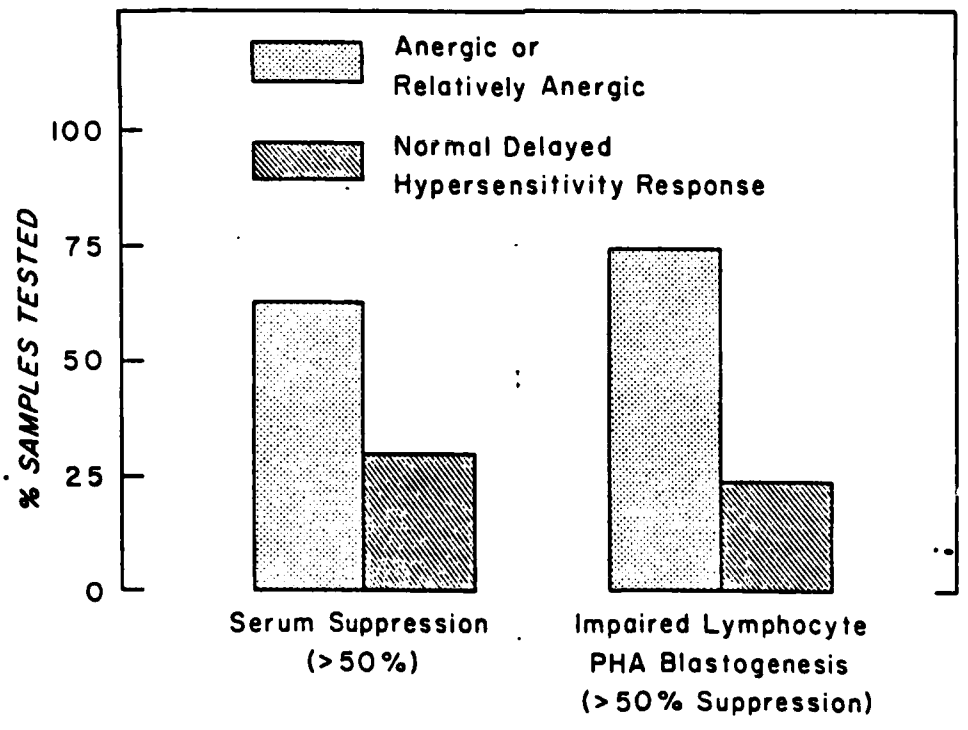


Figure 2: Demonstrated the serum suppressive activity and delayed hypersensitivity responses in two patients over time in the post burn period. Patient I had a 40% burn from which he recovered, patient II had a 30% burn which proved fatal. The persistance of anergy and significantly immunosuppressive serum in the patient with the fatal burn is noteworthy.

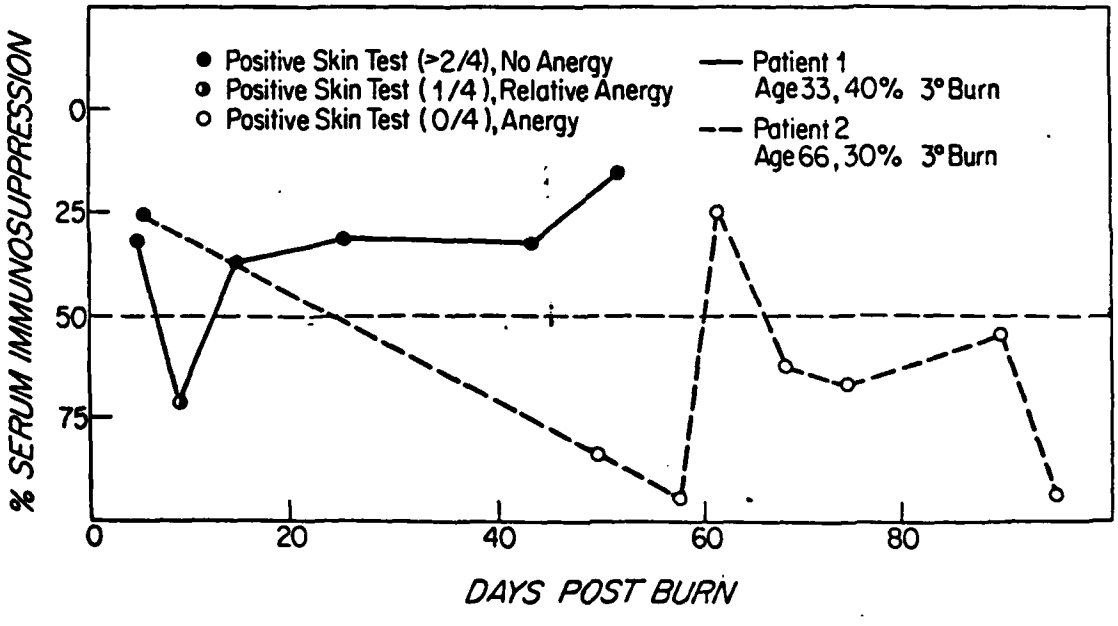


Figure 3 Pooled splenocytes from A/Jax mice that had been injected with 5 mg G-25 Peak 4 from the serum of a burned patient at day 0 were harvested at serial intervals after injection. These splenocytes were than added in graded dosages to 5 x 10^5 normal A/Jax splenocytes which were then exposed to an optimal stimulatory dose of purified PHA. The PHA response, measured as cpm H_3-thymidine incorporation, was compared with the response of 5 x 10^5 normal splenocytes alone.
It is apparent that splenocytes from peak 4 injected mice markedly suppressed the response of normal splenocytes to mitogen stimulation. Suppression appeared to be maximal at days 4 and 5 with loss of suppressor activity by day 12 after injection.

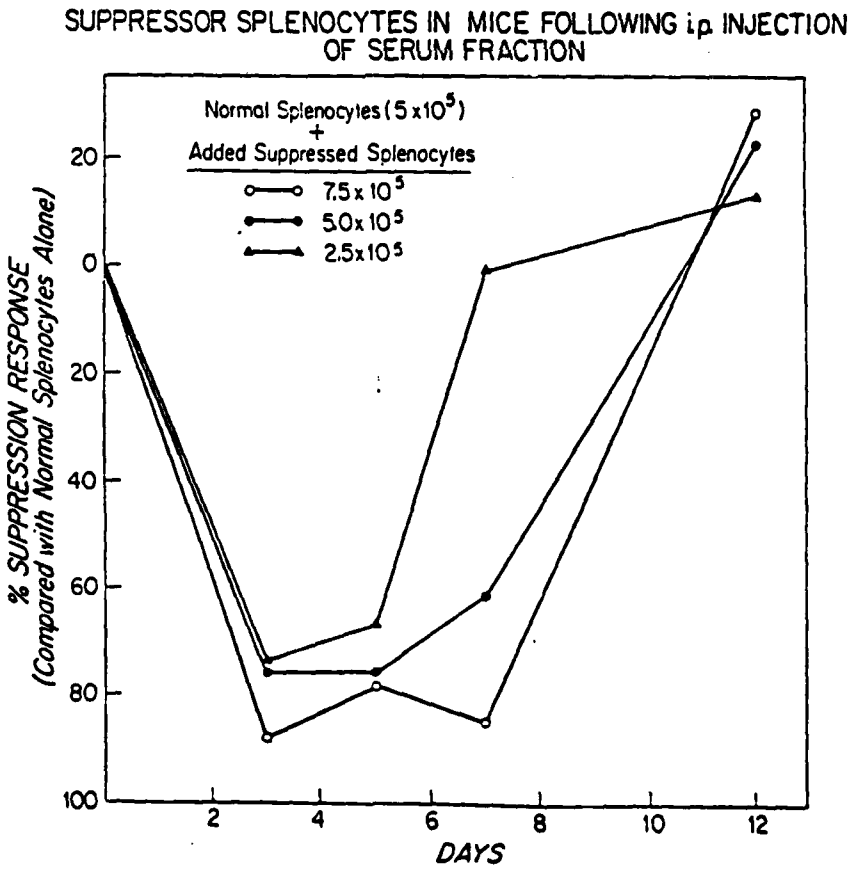


Figure 4 Analytical high-voltage electrophoresis of Sephadex G-25 Peak 4 from pooled trauma patients' serum (PTS), pooled burn patients' serum (PBS) and pooled minor trauma patients' serum (PCTS). Stained with ninhydrin. The ninhydrin positive moiety, slightly more basic than the lysine marker (K), found in trauma patients' serum and burn patients' serum but not in the serum from minor trauma patients or from normal volunteers contained the majority of the immunosuppressive activity when recovered by preparative high-voltage electrophoresis in one or two fraction cuts and tested for its ability to inhibit PHA-induced blastogenesis of normal human peripheral blood lymphocytes (see McLoughlin et al in Appendix), to inhibit the generation of the cytolytic cells in mouse mixed lymphocyte cultures (see Table I in Appendix), and to inhibit the plaque-forming cell response to SRBC in the Mishell-Dutton system (see Table I in Appendix).

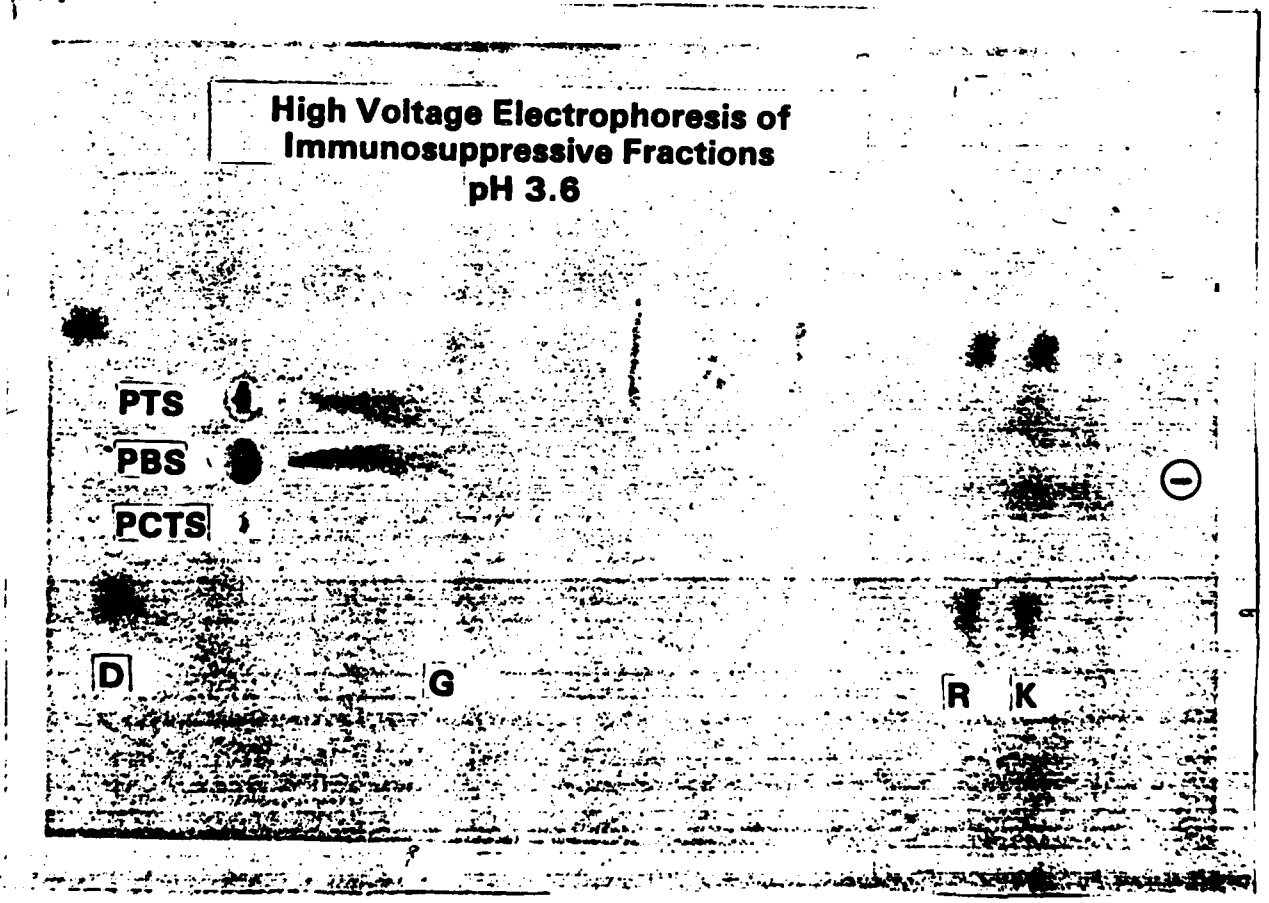


Table I

Effect of Serum Fractions

Source of Serum	% Suppression Normal Human Lymphocyte PHA Response		Mortali infecti (% incr
	Peak 1	Peak 4	Peak 1
Aneurysmectomy Pt.	15	100	0
Aneurysmectomy Pt.	31	50	40
Burn Pt.	38	97	0
Pooled Burn	*+5	82	0
Pooled Trauma			0
Pooled Normal	+2	+24	0

Peaks 1 and 4 from G-25 Sephadex chromatography of patient tested at 1 mg/ml for suppression of the PHA response of ly from normal donors. 5 mg of the fractions were injected i. A/Jax mice which were then challenged 24 hr. later with 1 x Listeria monocytogenes organisms.

* + = stimulation

TABLE II

Induction of Suppressor Cells in Mice Injected with Patient Serum Fractions

No. and Source of Mouse Splenocytes	% Suppression** of Splenocyte PHA Response by Trauma Serum Fractions	
	Peak 1	Peak 4
1×10^6 injected*	49	92
5×10^5 normal + 7.5×10^5 injected	23	92
5×10^5 normal + 5×10^5 injected	18	93
5×10^5 normal + 2.5×10^5 injected	14	83
	by Burn Serum Fractions	
	Peak 1	Peak 4
1×10^6 injected	14	71
5×10^5 normal + 7.5×10^5 injected	+7***	88
5×10^5 normal + 5×10^5 injected	+33	53
5×10^5 normal + 2.5×10^5 injected	+10	24

*splenocytes from A/Jax mice injected six days previously with 5 mg. of peaks 1 or 4 from G-25 Sephadex chromatography of trauma or burn patient serum.

**Compared with 5×10^5 normal mouse splenocytes.

***+ = stimulation.

TABLE III

Effect of Fractions Eluted from High Voltage Electrophoretograms of Pooled Major and Minor Trauma Patient Serum (Sephadex G-25 Peak 4)

Fraction No.	% Suppression Mishell-Dutton Assay 0.1 ml.*	0.025 ml.	% Target Cell Lysis after MLC (concentration of fraction approx. 0.1 ug/ml)	
Pooled Major Trauma Serum				
+ 1	0	0		
2	21	0		
3	8	4		
4	27	7		
5	16	3	24.4	
6 **	47	22	2.7	$p < 0.01$
7 **	50	26	11.0	$p < 0.01$
- 8	21	2		
paper blank	9	0	30.5	
Pooled Minor Trauma Serum				
+ 1	26	0		
2	0	0		
3	13	0		
4	19	0		
5	9	9		
6	5	0		
7 **	21	0	34.6	
8 **	17	0	30.0	
9	17	0	43.0	
- 10	3	0		
paper blank	8	0	45.4	

** basic area near lysine marker - see fig. 1
* 0.1 ml. of approx. 1 ug/ml solution of fraction

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