Suppression of Leukocyte Chemotaxis in vitro by Chemotherapeutic Agents Used in the Management of Thermal Injuries

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Polymorphonuclear leukocytes from burned patients exhibit suppressed chemotaxis possibly related to the susceptibility of such patients to opportunistic infection. This study assesses the effect of normal serum upon burn-suppressed leukocytes and the effects of three commonly used topical chemotherapeutic agents upon the chemotaxis exhibited by granulocytes from normal controls. In vitro incubation with normal serum restored chemotaxis to normal in the suppressed granulocytes from burned patients. The serum factor responsible for this restoration was heat labile. Serum albumin alone did not exhibit this effect. Both mafenide and silver sulfadiazine suppressed the chemotactic function of granulocytes obtained from normal controls, while silver nitrate exhibited no such activity. Studies of the chemotactic function of control granulocytes after incubation with sera from burned patients yielded similar results; only the sera from patients treated with silver nitrate failed to suppress normal leukotaxis. The chemotactic impairment found in leukocytes from burned patients, however, while related to burn size and predictive of prognosis, did not vary with the agent used for the topical therapy. These data suggest the presence of a reversible intrinsic defect in leukotaxis consequent to burn injury, related to some factor deficient in burn serum. In addition, extrinsic impairment of normal granulocyte leukotaxis by two commonly used chemotherapeutic agents is demonstrated.

THE USE of topical chemotherapeutic agents to control bacterial invasion in extensive burn wounds has become an accepted method of treatment. Mafenide acetate (Sulfamylon®), silver nitrate and silver sulfadiazine (Silvadene®) are widely used agents for such topical treatment. The advantages and disadvantages of each agent have been described and their effectiveness in decreasing the incidence of pseudomonas burn wound sepsis is well established. Despite this decreased incidence of pseudomonas burn wound sepsis, the mortality from extensive thermal injury remains high. The use of topical chemotherapy has been associated with increased frequency of infection by other opportunistic organisms. Infection remains the most frequent cause of death in patients who have sustained large burns.

The susceptibility of burn patients to opportunistic infection is not understood. Several defense mechanisms are known to be impaired, including the epithelial barrier, immune mechanisms and leukocyte function. We have introduced an assay of leukocyte chemotaxis to assess one aspect of polymorphonuclear leukocyte function in thermally injured patients and have demonstrated an inverse correlation between leukocyte chemotaxis and burn size.[8] In addition, suppression of leukocyte chemotaxis has been predictive of mortality in burn patients. This study of the mechanism of chemotactic suppression following burn injury assesses the effects of normal serum and of topical chemotherapeutic agents upon granulocyte chemotaxis.

Materials and Methods

Preparation of Leukocytes

On the day of testing 10 cc of heparinized blood (200 units heparin per 10 cc blood) was collected in a glass syringe. The aliquant was placed in an equal volume of clinical dextran (6%) in physiologic saline solution, containing glucose (400 mg/500 ml) and heparin (20 mg/100

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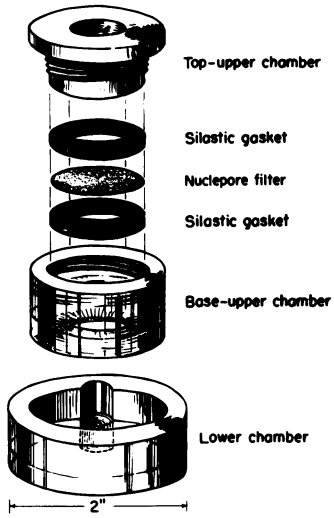

FIG. 1. Chemotactic chamber.

ml). Erythrocyte-mononuclear sedimentation was accomplished in a 50 ml conical tube at 37 C for 45 minutes. The leukocyte rich supernatant was removed with a Pasteur pipette and used within one hour of preparation. Average yields of 10^8 cells were obtained from 10 ml of blood. Ninety-nine per cent viability was confirmed by the trypan blue exclusion method after sedimentation.

Chemotactic Agent

Previous studies by Baum and Mouat[3] have demonstrated a mixture containing two parts of casein solution and one part of human serum to furnish reliable chemotactic attraction. Casein was prepared in a concentration of 5 mg/ml in Hank's solution. Human serum, type AB, from healthy donors was used throughout the study. The serum was obtained in the following manner: 1) 500 ml of peripheral blood was removed *via* the antecubital vein and placed in 50 ml conical tubes and allowed to clot at room temperature; 2) After clot retraction, the sample was centrifuged at 2000 *g* and the serum removed; 3) The serum was stored at −73 C in 3 ml aliquots.

Evaluation of Leukocyte Chemotaxis

Perspex chambers similar to Boyden's design were constructed (Fig. 1). Two ml of leukocyte-rich supernatant were diluted with 8 ml of Hank's solution and 2 ml of this mixture, containing approximately 4×10^6 cells, were placed in the upper chamber and 1.5 ml of chemotactic agent (casein-serum mixture) were placed in the lower chamber. A nuclepore* filter, 0.5 μ pore size, 25 mm diameter was interposed between the upper and lower chambers. The chamber was incubated at 37 C for 120 minutes. The nuclepore filter was removed, stained with Wright-Giemsa stain for 4 minutes, Wright's buffer for 4 minutes, cleared with xylene and mounted with xylene-permount (3:1) ratio on a glass slide. The nuclepore filter was examined microscopically under high power and the cells migrating through the nuclepore filter (bottom side) were counted as were the cells remaining on the starting side (top side) in each microscopic field. A total of 400 cells was counted. Chemotaxis was evaluated in the following manner, negating the necessity to count the cells before placement in the upper chamber.

$$\text{Chemotactic index} = \frac{\text{Number cells (attracting side)} \times 1000}{\text{number cells (starting side)}}$$

Leukocytes from thermally injured patients were compared with cells from normal healthy volunteers and the functional chemotactic index expressed as the per cent of the chemotactic index of the control leukocytes:

$$\text{Functional chemotactic index} = \frac{\text{chemotactic index burn patient}}{\text{chemotactic index normal volunteer}} \times 100$$

Duplicate chambers were used for each patient sample and simultaneous control samples were also performed in duplicate.

Preparation of Patient Serum

On the morning of each test, 10 cc of peripheral blood was obtained and allowed to clot. After clot retraction the sample was centrifuged at 2000 *g* and the serum removed. The serum was stored at −73 C or used within one hour of preparation.

Experimental Group

Studies were performed on 46 thermally injured patients with a mean burn size of 50.9% of total body surface (range 14.5–92.0%). The overall mortality among these patients was 65.2%. The mean burn size in those patients who expired was 59.0% (range 31.5–92.0%) while the surviving patients had an average burn size of 35.9% (range 14.5–70.5%). Autopsy examination was performed on 25 of the 30 nonsurviving patients. The mean day of death was 14.9 days (range 3–38 days). Among the autopsied cases, infection as the major cause of death occurred in 23 of the 25 cases (92.0%). Septic complications included pneumonia, burn wound sepsis, and septicemica. The mean day of clinical diagnosis of the septic complications was 9.8 days (range 3 to 19 days). None of the 16 surviving patients had systemic sepsis.

The patients' cells were tested at admission and then weekly until discharge or expiration. The patients were treated with topical mafenide acetate 10% ointment (Sulfamylon®), silver nitrate 5% solution in dressings, or silver sulfadiazine 1% cream (Silvadene®) using standard procedures of the Institute of Surgical Research.

*Nuclepore Corporation, 7035 Commerce Circle, Pleasanton, California 94566.

Evaluation of Effect of Burn Serum on Leukocyte Chemotaxis

The leukocyte rich supernatant obtained from normal volunteers was incubated in 1:1 ratio for 20 minutes at 37 C with serum obtained from thermally injured patients, normal serum obtained from healthy type AB donors or Hank's solution. This suspension was then diluted with Hank's solution and 2 ml of this mixture, containing approximately 4×10^6 cells were placed in the upper compartment of the chamber for evaluation of leukocyte chemotaxis.

Evaluation of Effect of Topical Chemotherapeutic Agents on Leukocyte Chemotaxis

The topical agents were dissolved in Hank's solution and incubated with 2 ml of leukocyte rich supernatant obtained from normal volunteers with final concentrations of: mafenide acetate 10 mg%, and 100 mg%, p. carboxybenzenesulfonamide 10 mg%, sulfadiazine 5 mg%, sodium sulfadiazine 5 mg%. In addition acetazolamide (Diamox®), 50 μg/ml and 100 μg/ml and gentamycin (Garamycin®) 4 μg/ml were also included in duplicate chemotactic chambers. Hank's solution without added drug was used in the same manner for control studies, which were performed in duplicate.

Evaluation of Effect of Dialyzed Burn Serum on Leukocyte Chemotaxis

Serum from thermally injured patients was dialyzed in cellulose casing (Visking, medical grade 23/32)* with diphosphate buffer 0.15 M, ph 7.4 for 48 hours at 4 C. The dialyzed serum was then added to 2 ml of the leukocyte rich supernatant from normal volunteers in 1:1 ratio and the mixture incubated at 37 C for 20 minutes. After dilution with Hank's solution, 2 ml of the mixture, containing 4×10^6 cells, was placed in the upper compartment of the chemotactic chamber for evaluation of leukocyte chemotaxis. Normal AB serum was maintained at 4 C for 48 hours and then incubated in the same manner as a simultaneous control.

Evaluation of Effect of Normal Serum on Chemotaxis

Serum from normal AB donors was used to assess the effect of normal serum upon leukocytes from burned patients. AB serum was added to 2 ml of the leukocyte rich supernatant in 1:1 ratio and the mixture incubated at 37 C for 20 minutes. After dilution with Hank's solution, 2 ml of the mixture, containing approximately 4×10^6 cells, was placed in the upper compartment of the chemotactic chamber for evaluation of leukocyte

*Visking Company, Chicago, Illinois.

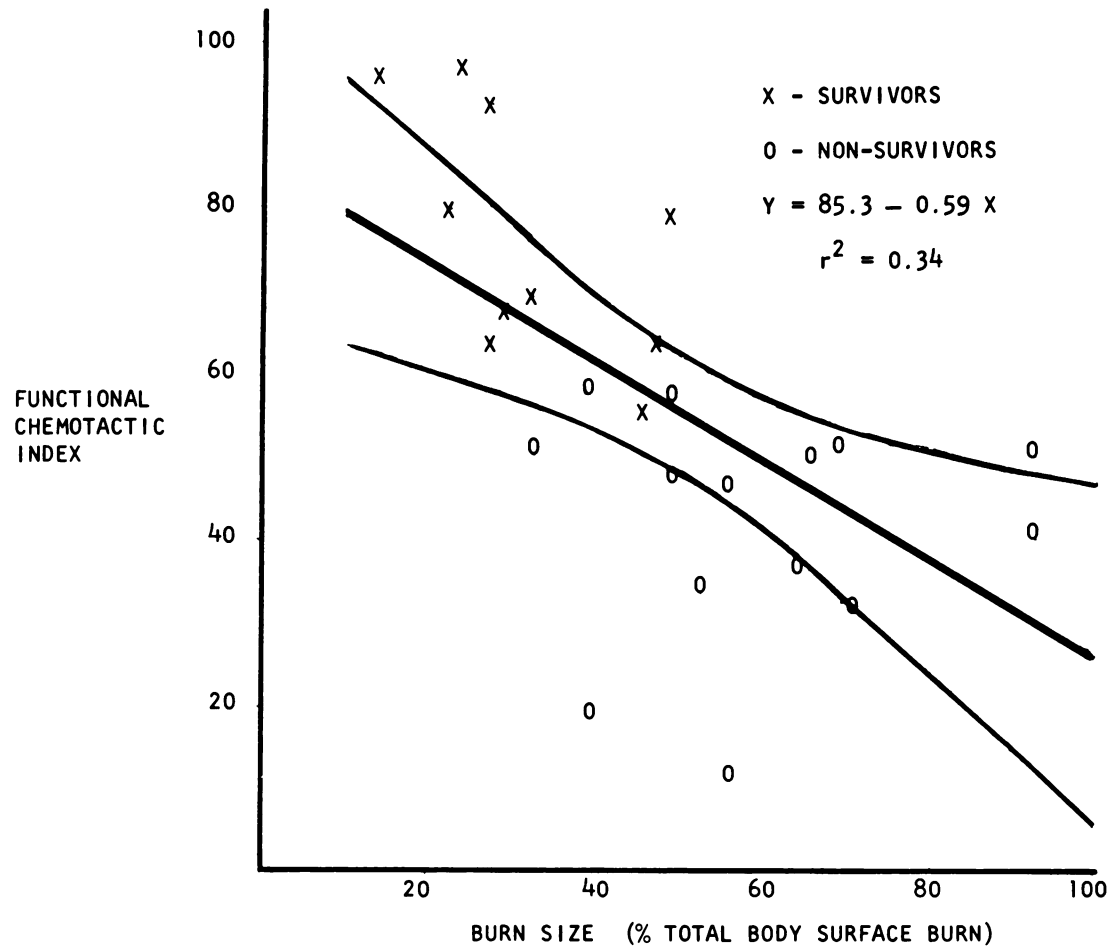

FIG. 2. Functional chemotactic index versus burn size during first 72 hours postburn.

TABLE 1. *Functional Chemotactic Index after 72 hours*

	Survivors	Non-Survivors
Patients	12	23
Determinations	36	34
Functional Chemotactic Index	97.7%	39.9%
Significance	P = < 0.01	
Range	61.2-130	14.6-75
Burn Size Mean	44.6%	57.9%
Range	25.5-70.5	31.5-92

TABLE 2. *Effect of Burn Serum on Normal Leukocyte Chemotaxis*

	Sulfamylon	Silver Nitrate	Silver Sulfadiazine
Number	27	3	14
Mean Functional Chemotactic Index	54.0%*	111.8%	42.4%*
Standard Error	±3.8	±5.8	±2.3
Burn Size Mean	46.3%	40.8%	65.0%
Range	18-70.5	35.5-48	30.5-96

*Significantly different from control, P < 0.01.

chemotaxis. Simultaneous controls were run using Hank's solution instead of serum.

Evaluation of Effect of Heated Normal Serum on Leukocyte Chemotaxis

Serum from normal AB donors was heated at 56 C in a water bath for 20 minutes. The leukocyte rich supernatant from thermally injured patients was incubated with the heated AB serum at 37 C for 20 minutes. This suspension was diluted with Hank's solution and 2 ml of this mixture containing approximately 4×10^6 cells were placed in the upper compartment of the chemotactic chamber for evaluation of leukocyte chemotaxis. As controls, Hank's solution and normal serum type AB were incubated in duplicate.

Evaluation of the Effect of Albumin on Leukocyte Chemotaxis of Thermally Injured Patients

Two ml of the leukocyte rich suspension obtained from thermally injured patients was incubated at 1:1 ratio with salt poor, human albumin* (25 g/100 cc) for 20 minutes. The suspension was diluted with 5 ml Hank's solution and 2 ml of this mixture (approximately 4×10^6 cells) was placed in the upper chamber of the chemotactic chamber for evaluation of leukocyte chemotaxis. As controls, incubation with Hank's solution and normal serum type AB was performed in duplicate.

Results

Chemotaxis in Thermally Injured Patients

As simultaneous daily control values, the chemotactic index was measured in a total of 44 normal healthy volunteers (ages 23–54 years). A mean value of 764.4 with 95% confidence limits of 750–780 was observed.

During the first 72 hours following injury, 24 patients were studied. Burn size and functional chemotactic index were inversely related, with a regression of y = 85.3 −

*Cutter Laboratories, Berkeley, California.

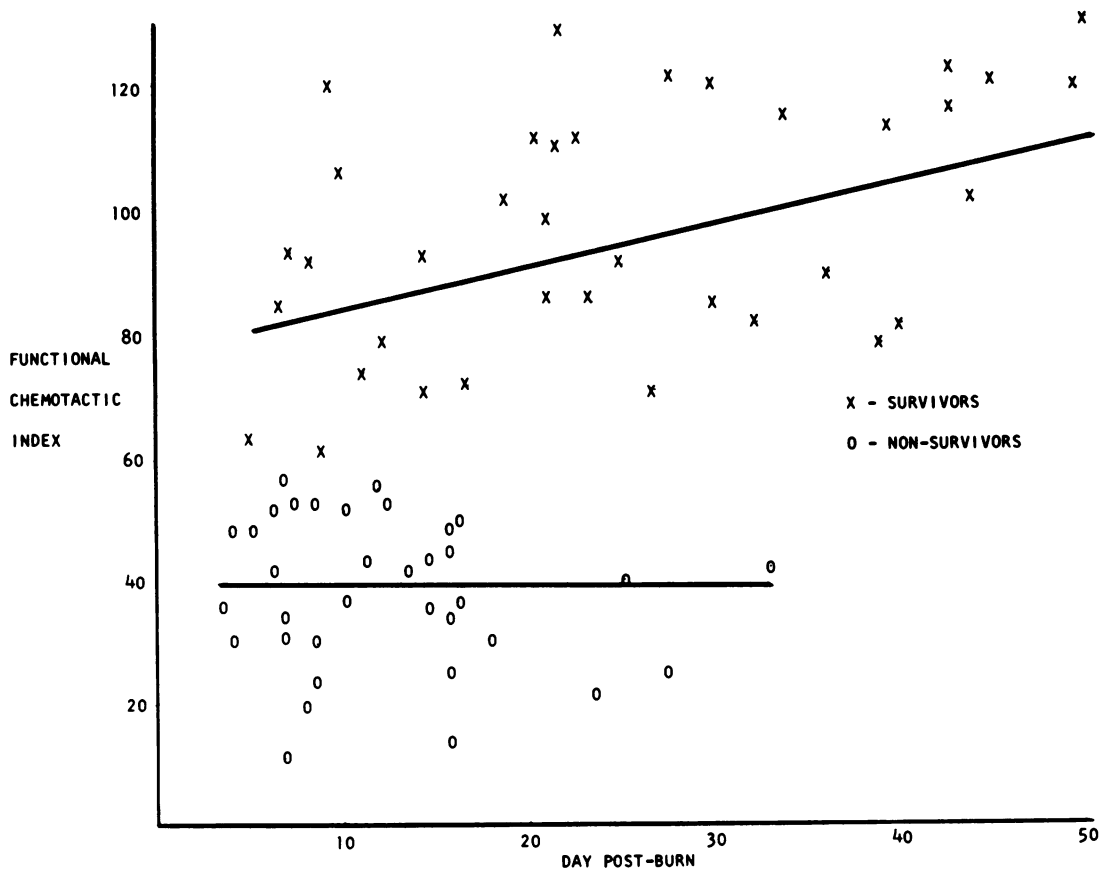

FIG. 3. Functional chemotactic index versus day postburn after 72 hours postburn.

TABLE 3. *Effect of Chemotherapeutic Agents in vitro on Normal Leukocyte Chemotaxis*

Chemotherapeutic Agent	Concentration	No.	Mean Functional Chemotactic Index
Mafenide Acetate	10 mg%	6	68.5% ± 1.9
Mafenide Acetate	100 mg%	6	54.7% ± 2.7
P-Carboxybenzene-sulfonamide	10 mg%	6	58.1% ± 2.0
Sodium Sulfadiazine	5 mg%	4	36.7% ± 2.0
Sulfadiazine	5 mg%	4	24.6% ± 3.6
Gentamycin	4 μg/ml	4	97.8% ± 7.4

TABLE 4. *Effect of Dialyzed Burn Serum on Normal Leukocyte Chemotaxis*

	Sulfamylon		Silver Sulfadiazine	
	Burn Serum	Dialyzed Burn Serum	Burn Serum	Dialyzed Burn Serum
Number	10	10	9	9
Mean Functional Chemotactic Index	43.7%	84.0%	42.0%	82.8%
Significance	P = < 0.001		P = < 0.01	
Burn Size Mean	50.2%		69.7%	
Range	18.0-70.5		30.5-96.0	

0.59 x (y= functional chemotactic index, x = burn size), and $r^2 = 0.34$ (Fig. 2).

After 72 hours, the patients separated into two groups, survivors and nonsurvivors, with surviving patients demonstrating improvement in leukocyte chemotactic function during their hospital courses (Fig. 3). The nonsurviving patients demonstrated neither significant increase or decrease in leukocyte chemotaxis from admission until death. The average functional chemotactic index in the surviving groups was 97.7, SE ± 2.3; whereas the average in the nonsurviving was 39.9, SE ± 2.3 (Table 1). No patient with a functional chemotactic index below 60 beyond 72 hours after injury survived; only one patient with an index greater than 60 expired. Statistical comparison revealed a significant difference between the mean indices of these groups (P<<0.01).

Diminished chemotactic function was observed prior to clinical infection in all patients who ultimately succumbed to infection. The onset of clinical infection was not associated with any change in the functional index. Functional chemotactic index did not vary with either the agent used for topical chemotherapy or with other identifiable differences in treatment. Age and sex were also without effect.

Effect of Burn Serum on Normal Leukocyte Chemotaxis

Serum obtained from 27 burn patients treated with mafenide acetate with a mean burn size of 46.3% total body surface (range 18.0–75%) produced significant suppression (P<<0.01) of chemotaxis of normal control leukocytes, decreasing the chemotactic index to 54.0% of control values (Table 2). The serum of 14 burn patients treated with silver sulfadiazine (mean total body surface burn 65.0%, range 30.5–96.0%) also suppressed normal control leukocyte chemotaxis to 42.4% of control (P <<0.01). However, the silver nitrate treated patients' serum produced no suppression of normal leukocyte chemotaxis although burn size was similar to the mafenide acetate treated group. Simultaneous control AB serum had no effect on normal leukocyte chemotaxis.

Effect of Topical Chemotherapeutic Agents on Normal Leukocyte Chemotaxis

Mafenide acetate and its metabolite, p-carboxybenzenesulfonamide at a physiologic concentration (10 mg %) usually obtained during treatment of major thermal injuries produced significant suppression (P <0.01) of normal control leukocyte chemotaxis to 68.5% (SE ±1.9) and 58.1% (SE ± 2.0) respectively, of baseline values (Table 3). Increasing the concentration of mafenide acetate to 100 mg% resulted in a more pronounced depression (54.7% SE ± 2.7) of normal leukocyte chemotaxis (P <0.01). Sulfadiazine and sodium sulfadiazine at concentration of 5 mg % produced marked suppression of normal leukocyte chemotaxis to 24.6% and 32.8% respectively. Gentamycin at a concentration of 4 μg/ml produced no significant change of normal leukocyte chemotaxis. The suppressions exhibited by adding the topical agent directly to the cell suspension are similar to the suppressions obtained by incubating with burn serum from thermally injured patients treated with these agents.

Effect of Acetazolamide on Normal Leukocyte Chemotaxis

Since mafenide acetate (Sulfamylon®) inhibits carbonic anhydrase, the effect of acetazolamide (Diamox®) on normal leukocyte chemotaxis was also evaluated at concentrations of 50 μg/ml and 100μg/ml. Animal carbonic anhydrase inhibition occurs at serum levels of 50 μg/ml.

TABLE 5. *Effect of Normal Type AB Serum on Leukocyte Chemotaxis of Thermally Injured Patients*

	Baseline	Normal Serum Incubation
Number	33	33
Mean Functional Chemotactic Index	61.1%	107.7%
Standard Error	±5.7	±4.8
Significance	P = < 0.01	
Burn Size	46.8% (Range—14.5-70.5)	

TABLE 6. *Effect of Heated AB Serum on Leukocyte Chemotaxis*

	Baseline	Normal Serum Incubation	Heated Serum Incubation
Number	6	6	6
Mean Functional Chemotactic Index	70.6%	102.6%	72.5%
Standard Error	±8.8	±3.3	±10.7
Significance*	——	P = < 0.05	N.S.
Burn Size		50% (Range 32-63%)	

*Multiple comparisons with Baseline (Scheffe Test).

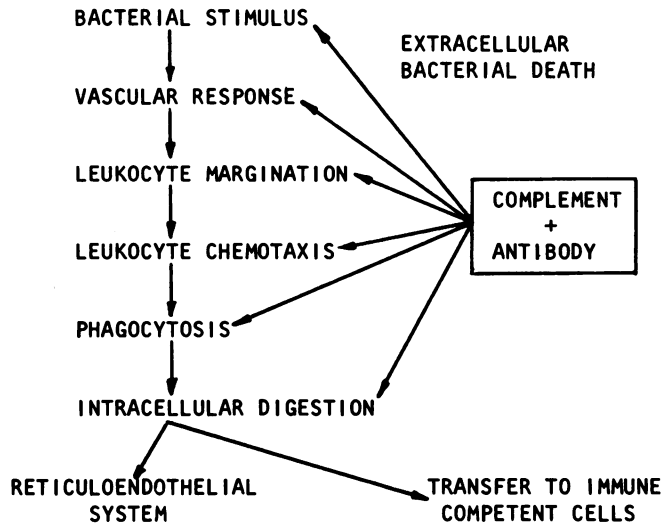

FIG. 4. Host defense mechanism against bacterial invasion.

Acetazolamide at 50μg/ml produced no suppression of normal leukocyte chemotaxis; whereas at 100μg/ml there was slight depression, to 85.2% of control ($P < 0.01$). We conclude that carbonic anhydrase inhibition by mafenide acetate was not responsible for the depression seen with burn serum or mafenide acetate.

Effect of Dialyzed Burn Serum on Normal Leukocyte Chemotaxis

Non-dialyzed burn serum obtained from 10 patients treated with mafenide acetate with a mean burn size of 50.2% total surface (range 18.0–70.5%) produced suppression of normal leukocyte chemotaxis to 43.9% of control values. Dialyzing the burn serum for 48 hours with a phosphate buffer significantly reduced ($p \ll 0.01$) the degree of suppression by burn serum to a mean value of 84.0% (Table 4). Dialyzed burn serum obtained from nine patients treated with silver sulfadiazine (mean burn size 69.7%, range 30.5–96%) also produced significantly ($P \ll 0.01$) less suppression than did the non-dialyzed burn serum with mean values increasing from 42.0% to 82.8%. These studies suggest that dialysis removed a chemotaxis-suppressing substance or substances, presumably the topical therapeutic agents mafenide acetate and silver sulfadiazine.

Effect of Normal Serum on Burn Leukocyte Chemotaxis

In 33 patients with a mean burn size of 46.8% total body surface area (range 14.5–70.5%) and having a mean baseline chemotactic index of 61.1% (SE ± 5.7) there was a marked increase of leukocyte chemotaxis after incubation with normal AB serum (Table 5). Levels increased to 107.7% (SE ± 4.8) of simultaneous normal controls Normal AB serum incubated with normal cells had no effect on leukocyte chemotaxis. The improvement of chemotaxis by normal serum was not related to the agent being used for topical chemotherapy and was not a dilution effect, since similar dilution with Hank's solution in the control runs had no effect.

Effect of Heated AB Serum on Leukocyte Chemotaxis

Six patients with a mean burn size of 50.0% total body surface burn (range 32–63%) and a mean functional chemotactic index of 70.6% (range 35.6-119%) demonstrated a significant ($P < 0.05$) increase of functional

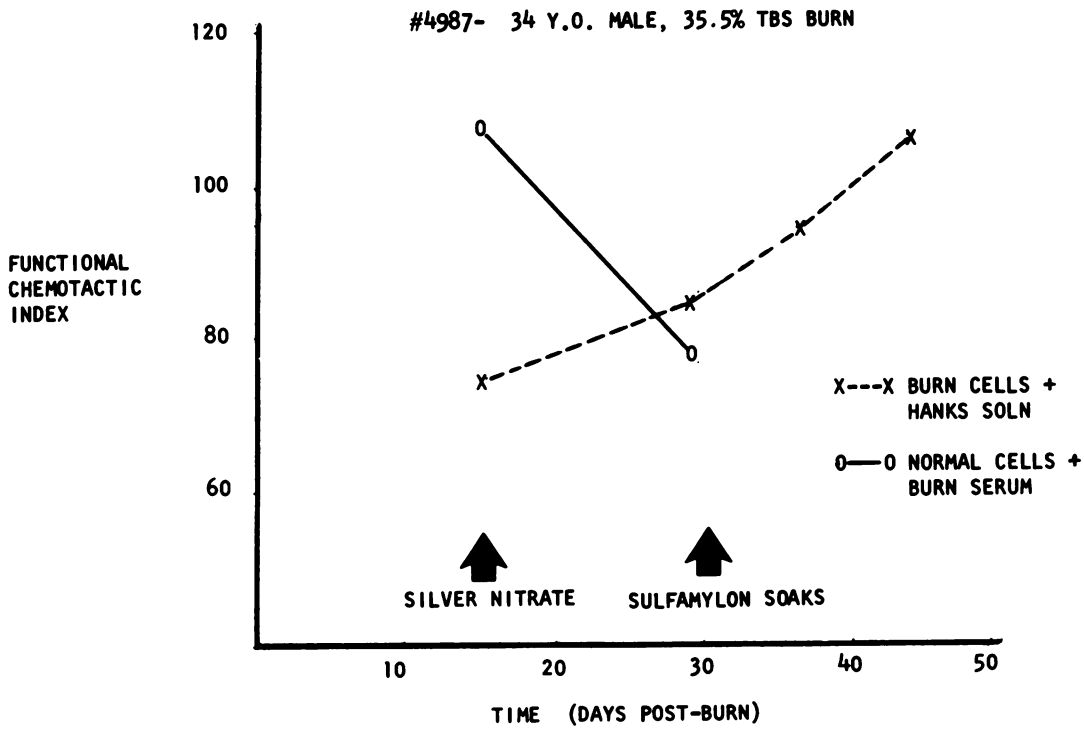

FIG. 5. Functional chemotactic index versus day postburn demonstrating suppression of normal leukocyte chemotaxis by burn serum after beginning mafenide acetate topical therapy.

chemotactic index (mean 102.6%; SE ± 3.25; range 93-115%) when their leukocyte rich suspension was incubated with normal type AB serum. However, when the normal type AB serum was heated for 20 minutes at 56 C, cooled at 37 C and then incubated with leukocyte rich suspension, there was no increase or decrease in the functional chemotactic index (mean 72.5%; range 41.3–106.7%) (Table 6). This experiment suggests that the factors in normal serum responsible for improving the chemotaxis of leukocytes from thermally injured patients are heat labile.

Effect of Albumin on Leukocyte Chemotaxis

Four patients with a mean burn size of 26.5% total body surface (range 20–30%) and a mean functional chemotactic index of 86.4% (range 44.9-119.4%) demonstrated no significant increase in leukocyte chemotaxis when their leukocytes were incubated with salt poor human albumin. The mean functional chemotactic index after incubation with salt poor albumin was 74.8% (range 35.3–111.2%). Although the albumin used was commercial salt poor albumin, this experiment suggests that the albumin fraction is not the active factor in normal serum responsible for increasing chemotaxis of leukocytes from thermally injured patients.

Discussion

Chemotaxis is an important biologic phenomenon determining the direction of motion of bacteria, plant and animal cells in reproduction, nutrition, cellular organization and inflammation. Various methods have been used to study chemotaxis but the method devised by Boyden in 1962[4] based on the ability of leukocytes to migrate through a filter toward a chemotactic agent, has been the most useful because of its simplicity and reproducibility. A number of modifications of this technic have been devised.[6,7,9] The importance of chemotaxis in the function of polymorphonucleocytes is demonstrated in Fig. 4 since even leukocytes fully capable of ingesting and killing microorganism are incapacitated if unable to sense and be directed towards the invading pathogen.

This study demonstrates 1) an intrinsic defect in the chemotaxis of leukocytes from burned patients and 2) suppression of the chemotaxis of normal leukocytes by serum from burned patients treated with either mafenide acetate or silver sulfadiazine.

Chemotaxis is suppressed soon after burn injury and this suppression is proportional to the extent of injury. That this suppression is not a drug effect is indicated: 1) by its occurrence in patients treated with silver nitrate; 2) by its reversal upon incubation with normal serum, an effect removed by heating the serum; and 3) by the failure of dilution alone to duplicate the effect of normal serum. In addition, the serum from one patient did not suppress the chemotaxis of normal cells during treatment with silver nitrate but inhibited such chemotaxis during treatment with sulfamylon soaks (Fig. 5). The chemotaxis of the patients' own cells was suppressed during both forms of treatment and subsequently returned to normal.

The effects of mafenide acetate and silver sulfadiazine upon normal leukocyte chemotaxis are readily demonstrated at concentrations which occur in serum during treatment. Silver nitrate does not exhibit such an effect in vitro.

Susceptibility to opportunistic freedom is a hallmark of burn injury. The etiology of this susceptibility is not understood, but one proposed explanation is failure of granulocyte defense. Others have demonstrated functional impairment of granulocytes from burned patients[1,2,5] and this study suggests that delivery of these impaired granulocytes may also be faulty. The study further suggests a dilemma: the use of either mafenide or silver sulfadiazine to protect the burn wound from infection may cause further suppression of the delivery of granulocytes and thereby enhance the susceptibility of the patient to infection by other routes. Such a hazard of therapy has not been examined previously and its importance is not defined at this time.

Incubation in normal serum restores chemotactic function to the granulocytes obtained from burned patients. This restoration of function appears due to a heat labile component of normal serum. If similar restoration of function can be achieved in vivo, this observation may have clinical importance, since such restoration would permit a direct test of the relationship between chemotactic suppression and susceptibility to opportunistic infection in burned patients.

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