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Neutrophil Function in Anergic Surgical Patients:

Neutrophil Adherence and Chemotaxis

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Skin test anergy (A) to recall antigens identifies surgical patients at high risk for sepsis. We studied neutrophil function in such patients to assess any alteration in their host defense mechanisms. Neutrophil adherence was measured with a modified adherence assay capable of measuring the adherence of neutrophils in whole blood or purified neutrophil suspensions, and neutrophil chemotaxis was assessed by the Boyeden technique. Twenty-one laboratory controls had a neutrophil adherence of 71.5 ± 3.8% (mean ±SD) and chemotaxis of 128.1 ± 2.4 μ (mean ±SD). Fifty-four hospitalized patients with normal skin tests had neutrophil adherence of 72.5 ± 13.1% ($p \sim 0.5$ relative to control) and chemotaxis of 123.3 ± 3.1 μ ($p \sim 0.5$). Twenty three relatively anergic patients had values of 84.3 ± 7.9% ($p < 0.001$) and 103.7 ± 2.0 μ ($p < 0.001$). Forty five A patients had adherence of 85.0 ± 7.0% ($p < 0.001$) and chemotaxis of 90.4 ± 2.9 μ ($p < 0.001$). The correlation coefficient between increased neutrophil adherence and decreased chemotaxis $r = 0.81$ has $p < 0.0005$. A factor which increased the adherence of normal control neutrophils was found in the plasma but not the serum of anergic patients. Inhibitors of control neutrophil chemotaxis have been shown in both serum and plasma of patients with decreased autologous neutrophil chemotaxis. We propose that this altered neutrophil function (possibly with other defects) in anergic patients may compromise their host defenses and render them susceptible to infection.

CUTANEOUS ANERGY TO RECALL skin test antigens, has been associated with an increased rate of sepsis and mortality in surgical patients.[9,10] The role of host defense mechanisms in preventing infection in such patients is being increasingly debated.[12] A complete study of all aspects of host defense against bacterial pathogens has not yet been done. The polymorphonuclear neutrophil (PMN) is the principle cell in defense against bacterial invasion. In the classic sequence of inflammation, PMNs first adhere to the vascular endothelium, with subsequent passage through the microvasculature endothelium by diapedesis and migration to the inflammatory focus along chemotactic gradients set up by the invading pathogen and local factors. Once at the site of bacterial invasion, PMNs ingest (phagocytose) and digest (kill) opsonized pathogens. There are no data on neutrophil adherence in patients with skin test anergy.

Modifying existing systems, the adherence of neutrophils from patients with various skin test responses was measured and correlated with PMN chemotaxis. Modulators of neutrophil adherence in serum and plasma were identified and the effects of several drugs on neutrophil adherence and chemotaxis were studied.

Materials and Methods

Patient Population

Surgical patients on the wards and the intensive care unit of the Royal Victoria Hospital were skin tested

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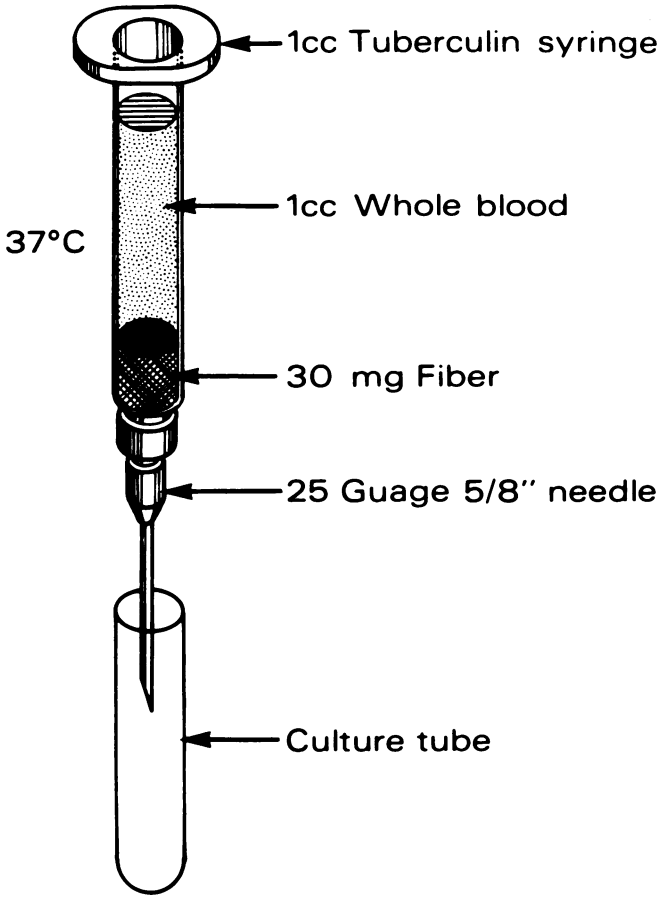

FIG. 1. A graphic representative of the neutrophil adherence assay system.

by the intradermal injection of five recall antigens: Candida, Mumps, PPD, Trichophyton, and Varidase.[10] All skin tests were administered and read by one person. A positive response was defined as induration greater than 5 mm seen at 24 and/or 48 hours. From this a normal (N) response was defined as two or more positive responses to any of the antigens at either reading: relative anergy (RA) as one positive response; and anergy (A) no response to any of the antigens. Patients were skin tested either preoperatively or postoperatively. Blood was drawn for neutrophil function after the 48 hour reading was obtained. The age range of the patients varied from 18 to 95 mean age 58 and the male/female ratio was 1.2:1.0. Patients had a variety of surgical diseases ranging from simple breast lumps requiring biopsy to carcinoma of the head of the pancreas requiring a Whipple resection. Twenty-one healthy laboratory personnel ages 20–55 were used as controls. The controls were asked to refrain from ingestion of any medication for 72 hours prior to study. All medications given to patients were noted.

Collection of Blood, Plasma, Serum and Preparation of Neutrophils

Blood was collected in 10 cc heparinized tubes (10 units Heparin/cc) by venopuncture and treated accordingly. Heparin was excluded when blood was collected for the purpose of obtaining serum. After clot retraction at 4° overnight, the serum was separated by centrifugation at 2,000 × *g* for 20 minutes. Similarly, plasma was collected after centrifugation of the particulate elements in whole blood. Neutrophils were prepared from whole blood as follows. Two cubic centimeters of Dextran-40 (Pharmacia) was added to 10 cc of heparinized whole blood and the red blood cells were allowed to sediment at 1 × *g* for 60 minutes at 37°. The leukocyte rich supernatant was then centrifuged at 200 × *g* for 10 minutes. The pellet was washed three times with minimal essential medium (MEM, Microbiological Associates) and resuspended to a final concentration of 3–5 × 10^6 neutrophils/cc. In order to obtain PMNs the leukocyte rich pellet was resuspended in a mixture of 1 cc plasma and 1 cc phosphate buffered saline and then layered onto ficoll-hypaque (Pharmacia). After 25 minutes centrifugation at 480 × *g* the PMNs were separated from the mononuclear cells, washed three times with MEM and resuspended in MEM at 3 × 10^6 PMN/cc.

Neutrophil Adherence

The assay system for whole blood consisted of a 1 cc tuberculin syringe fitted with a 25G, ⅝ inch needle standing in a 12 × 75 mm borosilicate glass tube in a rack (Fig. 1). The syringe was packed with 30 mg of nylon fiber (Leuko-Pack Leukocyte Filter, Code 4C2401, Fenwal Laboratories, Deerfield, IL). The fiber was prewetted with 1 cc of MEM pH 7.4 (MEM + 10 mM Hepes) and packed with a plunger into the bottom of the syringe barrel. This assembly was then prewarmed to 37° in an incubator. Simultaneously 3.5 cc of whole blood to be tested, was prewarmed to 37°. Using a 1cc tuberculin syringe fitted with a 16G, 3½ inch needle, 1 cc of whole blood was added to the top of the fiber column and allowed to percolate through. This took about 10 minutes at 37°. Each sample was done in triplicate. All readings were done by one person who was not aware of the source of the blood being tested. The cell count in the remaining blood not passed through the column was determined by means of an automatic cell counter (Coulter Counter Model Z_F, Coulter Electronics Inc.) The cell count was similarly determined in the three effluents and averaged. The neutrophil adherence was thus determined as a percentage using the formula:

$$\% \text{ Neutrophil Adherence} = \frac{\text{Control WBC} - \overline{\text{Effluent WBC}}}{\text{Control WBC}} \times 100$$

The adherence system for purified neutrophils was similar except that neutrophils were separated from whole blood as described above. While this was being done, a 1 cc tuberculin syringe as for the whole blood adherence assay was packed with 5 mg of fiber, accurately weighed on an analytical balance. The fiber was prewetted with MEM pH 7.4 and packed as tightly as possible at the bottom of the syringe. The columns, standing in borosilicate glass tubes and the cell suspension to be tested were prewarmed to 37°. The adherence of the purified neutrophil suspension was then determined similar to that for neutrophils in whole blood.

Neutrophil Chemotaxis

Chemotaxis was carried out using a modified Boyden technique[1] and the leading front method of Zigmond and Hirsch[15] in Sykes-Moore tissue chambers. Briefly, 150 μ thick 3 μ pore size filter (millipore) was used to separate the chamber into upper and lower compartments closed off by rubber gaskets and round glass coverslips. Using 25 G needles the attractant casein 5 mg/ml (Hamerstein) in MEM pH 7.4 was injected into the lower chamber and the neutrophils to be tested, prepared as before and resuspended in MEM pH 7.4, were added to the upper chamber. The chambers were incubated at 37° for 90 minutes. The filters were then removed, fixed and stained.[14] After clearing in xylene they were examined under high power and using a micrometer rack, the distance migrated by the leading-front cells into the filter was measured. Five random fields were checked for each filter and three chambers were examined for each experimental point. Chemotactic migration distance reported is the mean and standard deviating of these values, unless otherwise indicated. All the filters were read by one person in a blind fashion. Under these experimental conditions the PMN is the only cell type that shows any significant migration and is easily distinguishable by its multilobed nucleus.

Effects of Drugs, Plasma or Serum

The effect of hydrocortisone, ascorbic acid, ethanol, acetylsalicylic acid (ASA) and levamisole-HCl on PMN function was studied *in vitro*. The drug was added directly to an aliquot of whole blood at the desired concentration and then the adherence was determined after 30 minutes incubation. In cases where the drug alone was to be tested against neutrophils free of blood components, the drug was added to the final resuspending medium in most cases MEM, at the desired concentration.

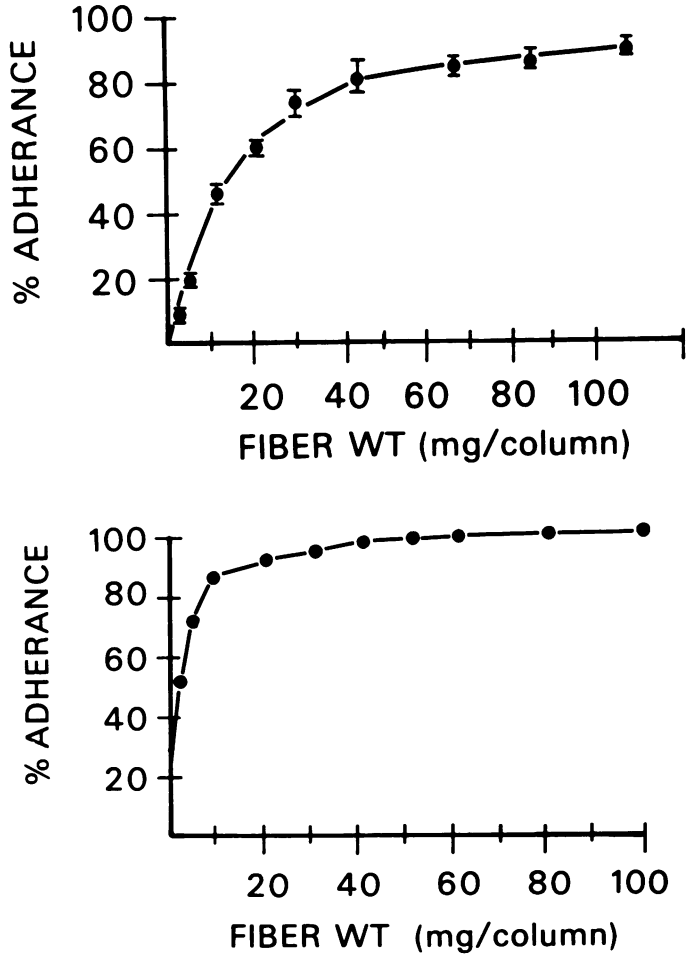

FIG. 2. The relationship between fiber weight and neutrophil adherence in the whole blood system (2a) and the purified neutrophil system (2b).

Purified neutrophils were also resuspended in plasma or serum from normal or anergic patients. After a 30 minute incubation the adherence was measured in the purified neutrophil system. A similarly treated aliquot of neutrophils was also assayed in the chemotaxis measuring system.

Results

Neutrophil adherence was proportional to the fiber weight in both systems and exhibited a nonlinear relationship (Figs. 2a and b). The fiber weight of 5 mg for the purified neutrophil system gave a control neutrophil adherence of 72.7 ± 5.7% (n = 21, mean ± SD).

TABLE 1. *Day to Day Variation in the Purified Leukocyte Adherence of Control Subjects*

Subject	Day 1	Day 2	Day 3	Day 4	Day 5
N.C.	76%	74%	71%	77%	72%
I.H.	72%	74%	72%	69%	—
J.M.	70%	73%	—	73%	75%

The comparable fiber weight for the whole blood system was found to be 30 mg and gave a control adherence of 71.5 ± 3.8% (n = 21). Variations in the cell concentration from 1×10^6 to 50×10^6 neutrophils/cc in the purified neutrophil system resulted in all adherence values falling within the 95% confidence limits of the control. Variation of the pH of the resuspending medium showed linear constant adherence between pH 6 to 7.8. The adherence became markedly decreased above pH 8.5.

Day to day variation in control subjects is shown in Table 1 for the purified adherence system. Similar results were obtained with the whole blood system. The effect of glycolytic inhibitors and cation chelators on adherence and chemotaxis of PMNs from controls and patients with skin test anergy is shown in Table 2.

In order to ensure that measurements of neutrophil adherence using the two systems was indeed representative of granulocyte adherence, the PMNs were separated from mononuclear cells using ficoll-hypaque gradients in six control subjects and then measurements of the adherence of these PMNs were made using 5 mg columns. The mean adherence was 73.7 ± 5.9%, which did not differ significantly from the mean laboratory normal control adherence, measured with the whole blood system (72.7 ± 5.7%), or the purified neutrophil system (72.4 ± 4.4%). Differential counts performed manually on five adherence samples gave a PMN adherence of 72.4 ± 5.1%.

TABLE 2. *Effect of Glycolytic Pathway Inhibitors on Leukocyte Adherence from Control and Anergic Surgical Patients (Purified Neutrophil System)*

Neutrophil Source	Treatment	Chemotaxis (μ)	Adherence (Per Cent)
Control	MEM	125.0 ± 0.28	67
Control	Potassium Fluoride 0.1 M	6.2 ± 0.14	31
Control	EDTA 0.1%	6.1 ± 1.0	18
Anergic	MEM	91.7 ± 0.42	89
Anergic	Potassium Fluoride 0.1 M	7.5 ± 0.42	22
Anergic	EDTA 0.1%	7.3 ± 1.3	17

Table 3 shows the cumulative results of adherence measurements with the whole blood system as well as simultaneous neutrophil chemotaxis in all subjects tested. The 21 laboratory controls had two or more different determinations (n = 54) during the course of this study and the mean adherence (±SD) of these readings was used as the laboratory control neutrophil adherence. Similarly the laboratory control of neutrophil chemotaxis was set at 128.1 ± 2.4 μ (mean ± SD). Fifty-four hospitalized patients who demonstrated normal skin test response had a slight reduction in neutrophil chemotaxis. Their adherence was 72.5 ± 13.1%. These patients showed a wide standard deviation because some of them had an evolving hospital course. Some deteriorated after they were studied, and some were recovering both their health and skin test response. Twenty-three relatively anergic patients had a significantly increased adherence from the laboratory normal control and the in-hospital normals, and similarly 45 anergic patients had significantly increased adherence compared to the laboratory controls and in-hospital normals ($p < 0.001$) but not the in-hospital relatively anergic population. Eighty-four surgical patients who had simultaneous measurements of neutrophil adherence and chemotaxis, are shown in Figure 3. The regression equation is $Y = 137.7 - 0.51X$ with a correlation coefficient, $r = 0.81$. This is highly significant with $p < 0.0005$.

The effect of serum and plasma on the adherence of purified normal control neutrophils was assessed using this system. In aliquots of 15×10^6 cells, MEM was removed by centrifugation and the cells were resuspended in 3.5 ml of normal control serum, normal control plasma, anergic serum, or anergic plasma. Serum and plasma from four different normal and anergic patients was used. One aliquot was resuspended in MEM only, as a control. After a 10 minute incubation the adherence was measured with 5 mg columns. Neutrophil chemotaxis was assessed simultaneously. The anergic serum donors (n = 4) demonstrated a mean

TABLE 3. *Relationship Between Whole Blood Neutrophil Adherence, Chemotaxis and Skin Test Response in Laboratory Controls and Hospitalized Surgical Patients*

Source of Blood	Number of Patients	Skin Test	Chemotaxis (μ)	Adherence %
Laboratory controls	21	N	128.1 ± 2.4	71.5 ± 3.8%*
Hospitalized	54	N	123.2 ± 3.1	72.5 ± 13.1**
Surgical	23	RA	103.7 ± 2.0	84.3 ± 7.9***
Patients	45	A	90.4 ± 2.9	85.0 ± 7.0***

* vs. *** $p < 0.001$, Student's t test.
* vs. ** $p \sim 0.5$, Student's t test.

autologous neutrophil adherence of 90.3 ± 1.3% and chemotaxis 93.7 ± 4.5 μ, both significantly abnormal. Table 4 shows that normal neutrophils resuspended in MEM showed the expected adherence of 72.3 ± 4.4% as well as normal chemotaxis. Resuspension in normal serum or serum from patients with skin test anergy demonstrated no significant changes in the adherence though the chemotaxis of the normal cells dropped to 94.7 ± 4.5 μ after treatment with anergic serum. Resuspension of normal neutrophils in normal plasma gave an adherence that was different from cells without treatment with plasma as measured with the purified neutrophil system. However, resuspension of normal cells in plasma from the four anergic patients demonstrated a significantly increased adherence of 74.5 ± 11.3% over the normal plasma treated cells and decreased chemotaxis of 93.3 ± 2.7 μ.

A number of drugs were investigated for their ability to alter neutrophil function *in vitro*. Hydrocortisone sodium succinate (Fig. 4) had no effect on concentrations from 0 to 5 mg/dl on either measurements from normal or anergic patients. Ascorbic acid (Fig. 5) at 0 to 5.0 mg/dl also had no effect. Ethanol (Fig. 6) at levels 0–1 g/dl was found to markedly affect both the chemotactic behavior and adherence of control and anergic neutrophils. Two other drugs were tested *in vitro*. Acetylsalycilic acid (ASA) at 0 to 12 × 10^{-5} M, was found not to alter neutrophil adherence measured with the whole blood system. The mean adherence of 11 patients (3N, 2RA, 6A) was 79.8 ± 8.5% before ASA treatment and 76.4 ± 8.6% after, which is not different ($p \sim 0.5$). When the PMN chemotaxis of patients with skin test anergy was measured after treatment with ASA *in vitro*, there was a partial correction of the defect (Fig. 7). Levamisole-HCl was added directly at 10^{-7} to whole blood. The mean whole blood adherence of 9 patients (1N, 4RA, 4A) was 80.2 ± 4.7% before levamisole treatment and 81.0 ± 6.5% after ($p \sim 0.5$). Individual patients also showed no significant differences. Neutrophil chemotaxis, however, was corrected to normal.

TABLE 4. *Effect of Treating Normal Control Neutrophils with Serum or Plasma from Normal Controls and Anergic Surgical Patients (Data are Mean ± SD of Treatment of Same Neutrophils with 4 Different Anergic Sera and Plasma Samples)*

Treatment of Cells	Chemotaxis (μ)	Adherence (%)
MEM	128.6 ± 2.6	72.3 ± 4.4
Normal serum	127.1 ± 2.4	68.7 ± 2.5*
Normal plasma	127.7 ± 1.8	24.0 ± 6.0**
Anergic serum	94.7 ± 4.5	68.8 ± 4.5*
Anergic plasma†	93.3 ± 2.7	74.5 ± 11.3**

* $p \sim 0.5$.
** $p < 0.001$.
† Mean adherence of the four anergic plasma donors was 90.5 ± 1.3%.

Discussion

There are three basic techniques to measure neutrophil adherence in whole blood. The technique described herein offers some advantages. Garvin's technique[5] is cumbersome and somewhat outdated. The technique of Bryant and Sutcliffe[2] is more refined and measures the adherence of leukocyte rich

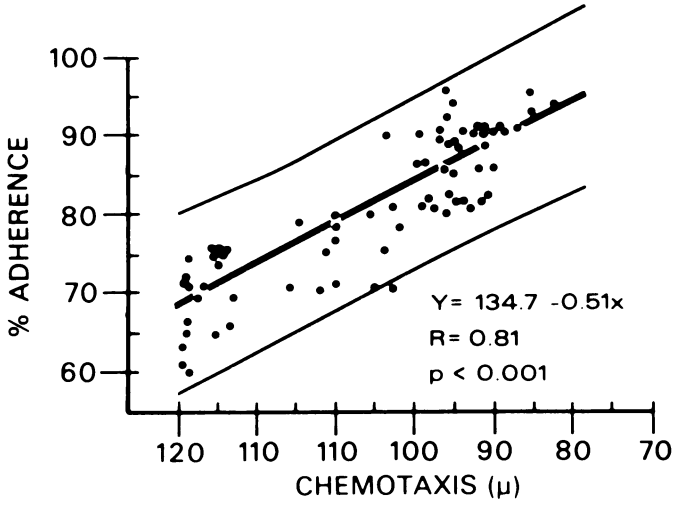

FIG. 3. The correlation between increased neutrophil adherence and decreased neutrophil chemotaxis in anergy. The regression line and 95% confidence limits are shown as dark solid and light solid lines respectively.

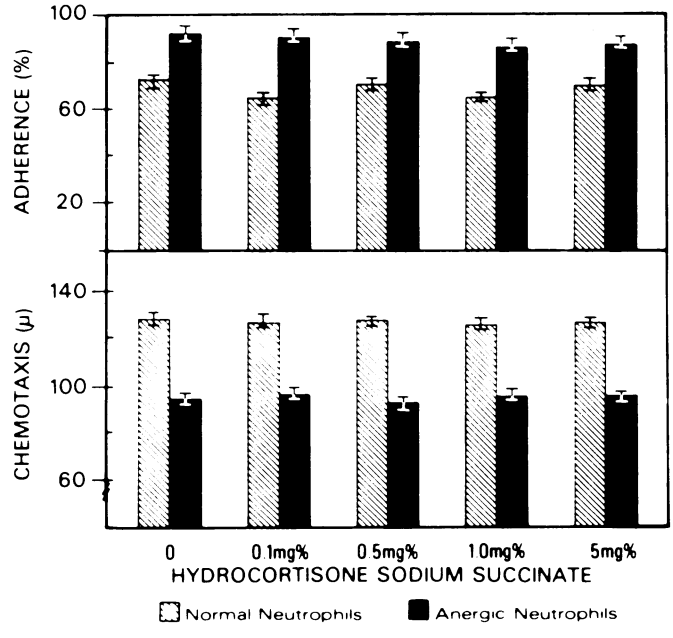

FIG. 4. The effects of hydrocortisone sodium succinate on the adherence and chemotaxis of neutrophils from normal and anergic patients.

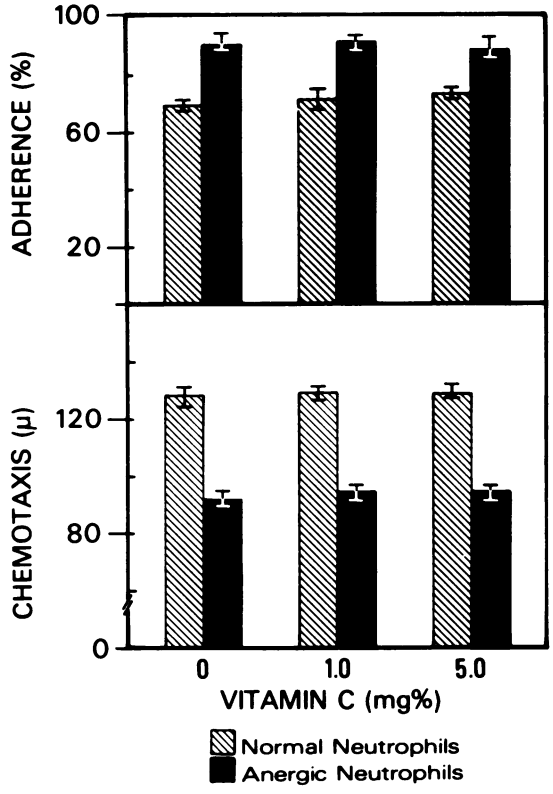

FIG. 5. The effects of ascorbic acid on the adherence and chemotaxis of neutrophils from normal and anergic patients.

plasma suspensions which corresponds to the "purified" leukocyte adherence in plasma reported herein. However, their technique does not allow for measurement of neutrophil adherence in whole blood. The technique of McGreggor et al.[7] and Strecher and Chinea[13] is similar in that nylon fiber columns are used, however, both measure neutrophil adherence in whole blood and not in purified neutrophil suspensions free of blood products. With the modified technique it is possible to measure both whole blood adherence *and* purified leukocyte adherence from the same individual for better comparisons, experimental flexibility and to insure that experimental artifacts such as that introduced by purification of the leukocytes or by the presence of other blood elements such as drugs or autologous serum have been minimized.

This technique is simple and requires readily available equipment. Great care must be taken to weigh accurately the 5 mg fiber aliquots. The flow rate is kept constant by means of the 25 G needle. The Coulter Counter allows up to 150 readings a day which means up to 50 patients or experimental points in triplicate can be tested. The day-to-day variation in controls is constant in both the whole blood adherence system and the purified leukocyte adherence system. The reliability of the method has been tested by introducing metabolic inhibitors to reproduce the data of previous investigators. With the type of fiber used, a linear relationship of fiber weight versus cell adherence as found by MacGreggor et al[7] could not be demonstrated. Rather, a curve resembling an enzyme kinetics plot was generated. The number of cells/column in the purified neutrophil system, a variable which is uncontrollable in the whole blood system gives constant adherence in the range used, between 1×10^6 and 50×10^6 cells/column ensuring reproducibility.

Using these modified adherence assays an increase in the adherence of neutrophils from patients with coexisting decrease in neutrophil chemotaxis and cutaneous anergy has been demonstrated. The association between cutaneous anergy and altered neutrophil function was not absolute. Previous work on trauma patients[11] demonstrated a two week "lag phase" between changes in skin test response and neutrophil function. Usually the skin test response returned to normal when the patient's condition improved and then improvement of chemotaxis followed approximately two weeks later. The same was observed here in sequential mesurements of ST response neutrophil adherence and chemotaxis. The high correlation between increased neutrophil adherence and decreased neutrophil chemotaxis has been demonstrated. Resuspension of washed normal control neutrophils in autologous plasma reduces their adherence from the value when the same cells are resuspended in MEM alone, indicating that factors exist in plasma that were washed off during the preparation of the cells which

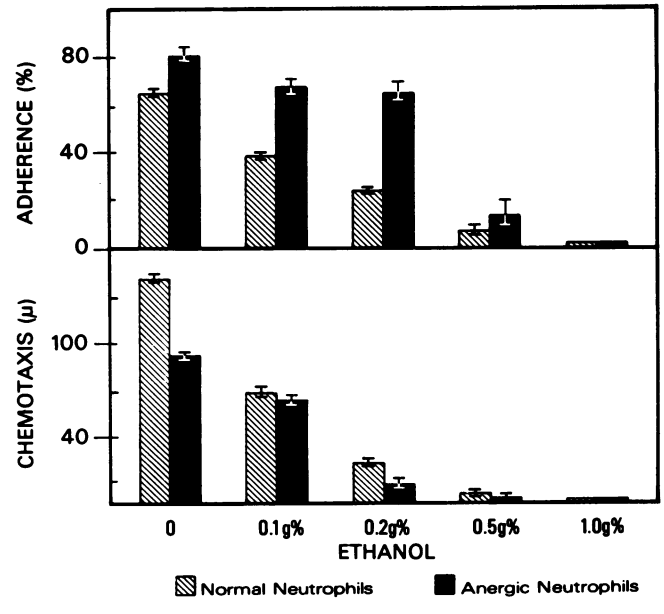

FIG. 6. The effects of ethanol on the adherence and chemotaxis of neutrophils from normal and anergic patients.

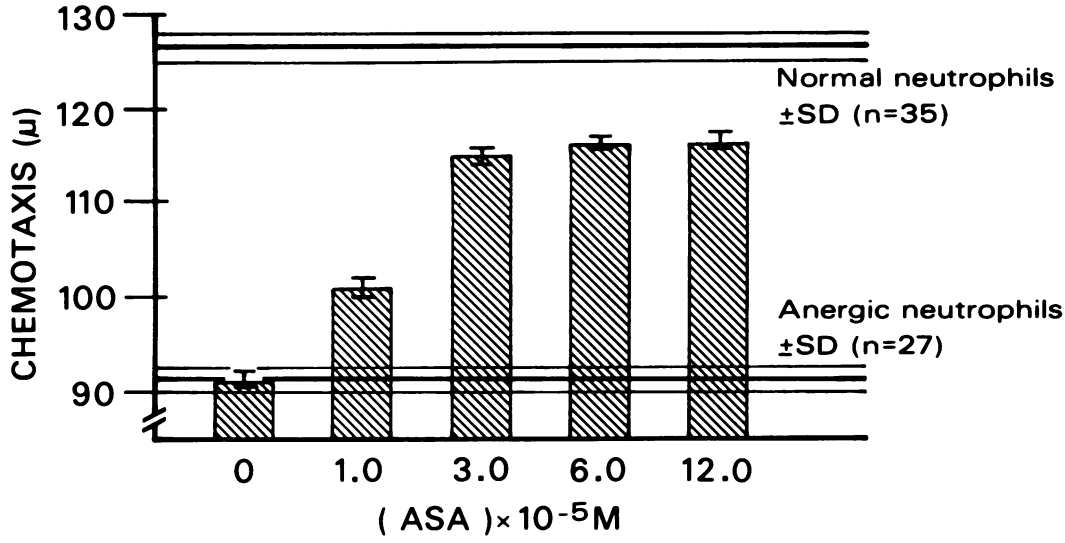

FIG. 7. The effect of acetyl salicylic acid on the chemotaxis of neutrophils from an anergic patient. Normal neutrophil chemotaxis was not altered. The mean ± SD of the normal laboratory control neutrophil chemotaxis and anergic neutrophil chemotaxis based on an earlier set of measurements is shown by the solid and light lines respectively.

are capable of modulating cell adherence. A factor exists in the plasma and not the serum of patients with increased neutrophil adherence which increases the adherence of normal heterologus neutrophils to nylon fiber. Similar findings of a plasma augmenting effect are reported by Bryant and Sutcliffe.[2] McGreggor[8] also reported increased granulocyte adherence in patients with inflammatory disease and localized the adherence augmenting factor in the plasma but not the serum of such individuals. Inhibitors of neutrophil chemotaxis, which are cell directed, are found both in serum and plasma of patients with decreased autologous neutrophil chemotaxis.[3] The factor which enhances normal neutrophil adherence is found only in the plasma of patients with increased autologous neutrophil adherence. The two modulators of neutrophil function must be different.

The effect of the various drugs on the adherence of neutrophils *in vitro* did not reveal any one agent suitable for clinical trials to modulate neutrophil function. McGreggor also obtained similar results with ASA and prednisome *in vitro*.[8] ASA may deserve *in vivo* trials since it also modulates neutrophil chemotaxis. However, caution must be exercised in giving this to severely ill patients, because of the risk of gastrointestinal hemorrhage and platelet effects. The immunomodulator levamisole, has been shown here and elsewhere[4] to significantly improve neutrophil chemotaxis *in vitro*. Though it has no effect on neutrophil adherence, it may be the best agent, from those available at this time to use in attempts to correct depressed neutrophil function *in vivo*.

The finding of cutaneous anergy, increased neutrophil adherence and decreased neutrophil chemotaxis in surgical patients has prognostic significance on clinical outcome. A study of 254 surgical patients with detailed sequential chemotactic measurements, demonstrated that the worse the chemotactic migration of neutrophils during a patient's hospital course, the higher was the rate of sepsis and mortality.[3] Similar findings using skin test anergy screening have been shown.[9,10] Assessment of PMN adherence is a simple determination. Its close relationship to skin test anergy and PMN chemotaxis make it an inexpensive and easy assay of PMN function. As such it may have applicability in centers where research PMN tests are not available.

The demonstration in this paper of increased neutrophil adherence which has a high correlation with decreased neutrophil chemotaxis is significant, in that this abnormality may play a role in altering the immune defense mechanisms of surgical patients perhaps preventing cells from getting into the extravascular space of the host. They may also be more adherent to the fine fibrilar lattice framework of the ground substance. Both these effects may serve to decrease delivery of appropriate cells to the inflammatory focus. It is also conceivable that these are normal responses of inflammation. The clinical finding however, of significant septicemia and abscess formation in patients with these defects is well established. The high association of the two though not proving a cause and effect relationship, is highly suggestive. Studies are underway at our institution to elucidate these points and to find means of modulating depressed neutrophil function *in vivo* in efforts to improve the dismal clinical course of such patients.

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