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Immunological Studies of Anergic Patients

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Sixty-one patients with a variety of different illnesses were studied with respect to skin test anergy and the presence of serum chemotactic inhibitors. In initial testing, 55% of the patient tests demonstrated negative skin test responses to all six test antigens. Sera from 65% of these anergic patients were capable of suppressing the migration of normal polymorphonuclear leukocytes toward chemotactic factors. Statistical analysis of the association of anergy and chemotactic inhibitory sera resulted in a P value of <0.0005. Chemotactic inhibitory sera were also capable of suppressing monocyte chemotaxis. No association of chemotactic inhibitory activity and lymphocytotoxic antibody or suppressors of mitogen-induced lymphocyte blast transformation were noted. In addition, T-cell populations in some anergic patients were studied by the erythrocyte-binding technique. Erythrocyte-binding lymphocytes in anergic patients were significantly suppressed when compared to normal controls, but not when compared to skin test-positive patients. The data presented here indicate a close parallel between skin test anergy and the presence of serum chemotactic inhibitory activity. The exact relationship is yet undefined but may indicate the involvement of chemotactic inhibitors as immunological regulators in the host during a variety of systemic illnesses.

Anergy has been defined as impaired or absent ability to react to specific antigens. This phenomenon has been described in a variety of illnesses including cirrhosis (26), systemic lupus erythematosus (14), lymphoproliferative disorders (8, 33), uremia (32), T-cell deficiency (11), various cancers (6, 16, 21, 29), tuberculosis (15, 18), myocardial infarction (J. Grossman, J. Baum, J. Glockman, J. Fusner, and J. Condemi, Clin. Immunol. **51**:127, 1974), viral infection (5, 12, 23), patients with leukocytosis (13; D. E. Van Epps, L. L. Palmer, and R. C. Williams, Jr., Clin. Res. **21**:509, 1973) and in patients after immunosuppressive therapy (19, 25, 29) or thoracic duct drainage (22). The cause of anergy associated with immunosuppressive therapy, thoracic duct drainage, or various lymphoproliferative disorders may obviously be the absence or malfunction of T cells. Anergy associated with diseases where T-cell malfunction has not been clearly defined remains poorly understood. In a previous study we demonstrated and characterized leukocyte chemotactic inhibitors associated with transient skin test anergy in patients with various systemic illnesses (28). Although the presence of serum chemotactic inhibitors has been previously observed (2, 7, 10, 24, 30, 31), an association with skin test anergy had not been previously demonstrated. The present study expands our observations to include a variety of different illnesses. In addition, our experiments demonstrate that skin test anergy did not correlate with lymphocyte blast transformation inhibitors (6; J. B. Couser and D. A. Horwitz, Arthritis Rheum. **16**:539, 1973), circulating lymphocytotoxic antibody, or a proportional reduction in T cells when compared to skin test-positive patients.

MATERIALS AND METHODS

Skin testing. Patients hospitalized on the medical services of Bernalillo County Medical Center, Albuquerque Veterans Administration Hospital, and Albuquerque Public Health Service Indian Hospital for a variety of different conditions were skin tested with a battery of six antigens including intermediate strength purified protein derivative, coccidioidin, trichophytin, mumps, candida, and streptokinase-streptodornase (13, 28). In some instances sensitization and challenge with dinitrochlorobenzene were performed. In all cases skin tests were measured at both 24 and 48 h with respect to induration. Anergy was defined if all skin test responses were 5 mm or less in diameter. Fifty-four of the 61 patients studied had been hospitalized with an acute systematic illness; the remaining seven patients were drawn from individuals with multiple fractures, extensive trauma, or postpartum states.

Preparation of leukocytes and chemotactic assay. Human leukocytes were prepared from peripheral blood of healthy adult donors. Blood was mixed with heparin, 10 U/ml, to prevent coagulation. Neutrophil preparations were obtained as previously described by plasma gel sedimentation of erythrocytes (1). Monocytes were prepared by Ficoll-hypaque centrifugation (4, 34). The band of mononuclear cells was collected, washed with minimal essential medium (MEM), and adjusted to a cell concentration of 10^7 cells per ml. This preparation contained from 19 to 29% monocytes.

Chemotactic factors used in this study consisted of normal serum derived from either a single donor or a pool of four to seven donors. Assays for chemotactic inhibitor utilized a mixture of 10% normal serum and 10% patient serum as previously described (28).

Neutrophil chemotactic assays were performed by using a modification of the Boyden technique (3) as previously described (1). Monocyte chemotaxis was performed in a similar fashion with the following modifications: (i) a Ficoll-hypaque mononuclear cell preparation was used as a cell indicator; (ii) a 5-μm membrane (Millipore Corp.) was used rather than a 3-μm membrane; (iii) a 3-h incubation was allowed instead of a 2-h incubation; (iv) MEM instead of Hanks balanced salt solution was used as an incubation medium; and (v) cell counts were determined by differential counts under oil immersion (100×). Variable proportions of polymorphonuclear leukocytes were present in the mononuclear cell preparations. These did migrate through membrane filters (Millipore Corp.) but could be easily identified and excluded from enumeration of monocytes. A mixture of 10% normal and 10% patient serum was used as a chemotactic attractant and compared to results obtained with 10% normal serum alone. Previous experiments have shown that as the control serum concentration was increased from 10 to 20%, chemotaxis was also increased. Twenty-five oil immersion fields were counted with respect to monocytes, and the average number of monocytes per five oil immersion fields was calculated. Due to the increased pore size of the membranes used in the monocyte chemotaxis, an MEM control for random mobility was necessary. The difference between chemotaxis using MEM in the lower chamber and that using MEM made 10% with serum was considered to be the actual chemotaxis.

Erythrocyte binding technique for determining T cells. The erythrocyte binding technique (E binding) for determining T-cell proportions was performed as previously described (9, 17, 20). This technique used human peripheral blood lymphocytes and sheep erythrocytes to determine the percentage of lymphocytes with receptors for sheep erythrocytes. In the human system these cells are considered to be T cells (9, 17).

Lymphocytotoxic antibody. The amount of complement-dependent lymphocytotoxic antibody was determined by the method of Terasaki et al. (27). In this assay lymphocytes from six normal donors were prepared by Ficoll-hypaque centrifugation and used as target cells. The percentage of kill was determined by dye exclusion using phase-contrast microscopy. Lymphocytotoxic antibody was considered positive when the average percentage of kill was greater than 15%.

Blast transformation technique. Lymphocyte blast transformation was performed as described by Griffiths and Williams (M. M. Griffiths and R. C. Williams, Jr., Arthritis Rheum., in press), measuring the incorporation of tritiated thymidine. One-milliliter cultures containing 10^6 cells obtained by Ficoll-hypaque centrifugation of peripheral blood from normal healthy adults were used as indicator cells. Phytohemagglutinin (PHA) was used as a mitogen in these experiments, and in all cases a PHA titration curve was run to determine the cellular response at various concentrations of mitogen. Dilutions ranged from 1:20 to 1:320 of a stock preparation of PHA (GIBCO, Grand Island, N.Y.). Combinations of normal cells with normal serum, anergic patient serum, or diethylaminoethyl-cellulose (DEAE)-isolated IgG immunoglobulin from anergic patients were tested to determine if a serum inhibitor of blast transformation was present in the sera of various patients with no demonstrable skin test response. In some experiments, fetal calf serum was used in addition to human serum to compensate for any factors possibly absent in patient serum but necessary for transformation. All data were expressed as a stimulation index and calculated by dividing the stimulated cell counts by comparable unstimulated control at the optimal PHA concentration.

RESULTS

Presence of skin test anergy in the patient population. Sixty-one patients hospitalized for a variety of different systemic illnesses were skin tested with a battery of antigens capable of eliciting a delayed-type skin test response. Serial studies were performed on three of these patients. Thirty-four of 64 tests in 61 patients showed skin test responses of 5 mm or less to all antigens. These patients were considered anergic. Previous studies performed on patients representing our general hospital population have shown that 91% showed a positive response greater than 5 mm to at least one of the battery of antigens used in the current study (13).

Relationship of anergy to the presence of a serum chemotactic inhibitor. All 61 patients were tested for the presence of anergy-associated serum chemotactic inhibitors (28). Chemotaxis tests were performed by mixing a solution of 10% normal serum and 10% patient serum in Hanks balanced salt solution and subsequently using it as a chemotactic attractant for normal peripheral blood neutrophils. Chemotactic counts using this mixture were compared to results obtained using 10% normal serum alone. If chemotactic counts were less than 70% of those achieved with 10% normal serum alone, the test serum was considered to

contain chemotactic inhibitor (Table 1). When the normal serum concentration in the lower chamber was increased from 10 to 20%, an increase was noted in the average number of cells per high-power field (×400). As a corollary finding, when serum from a patient with inhibitor was used as a chemotactic attractant, the resulting chemotaxis was negligible. When using a mixture of 10% patient serum and 10% normal serum, chemotactic counts were only 35% of that expected with 10% normal serum alone, indicating the presence of chemotactic inhibitors. Parallel experiments indicated that chemotactic inhibitory sera also were capable of inhibiting chemotaxis when purified $C3_a$, $C5_a$, or kallikrein were used as chemotactic attractants.

All patients tested were compared with respect to the maximal diameter of any skin test response and the degree of chemotactic inhibition expressed as a percent of positive control (Fig. 1). A horizontal line in Fig. 1 at the 5-mm mark indicates anergy. Anything below this line was considered a negative response. The vertical line at the 70% level represents the line of chemotactic inhibition. Any point to the left of this line indicated that the serum contained chemotactic inhibitor. Sixty-four tests are summarized in Fig. 1, since three of the 61 patients were followed through the course of disease and tested both at times when they were skin test positive and negative. Comparison of chemotactic inhibitors and skin test anergy (Fig. 1) shows that 22 of the 64 points fell in the quadrant representing anergy and the presence of inhibitor. It was of interest that 13 of 22 points in this quadrant indicated a totally negative response. Only two points fell in the quadrant representing chemotactic inhibitor and a positive response. One of these patients had a lymphoproliferative disorder and the other had gonococcal arthritis treated with high doses of penicillin. Twenty-eight of the 64 points fell in the noninhibitor skin test-positive quadrant, and 12 points fell in the noninhibitor skin test-negative quadrant. It should be noted that most of the 12 tests in the noninhibitor skin test-negative quadrant indicated some skin test reactivity, whereas only two points showed a totally negative skin test response. Statistical analysis of the relationship of anergy and chemotactic inhibitor resulted in a P value of less than 0.0005. This correlation constituted one of the major findings in the present study.

The presence of anergy or inhibitor was not associated with any one particular disease. A patient summary showing the diagnosis and the

TABLE 1. *Demonstration of serum chemotactic inhibition*

Chemotactic attractant	No. of cells per HPF per membrane[a]	Avg no. of cells per HPF
10% Normal serum	90,113	102
20% Normal serum	169,172	171
20% Patient serum	3,11	7
10% Normal serum + 10% patient serum	35,37	36

[a] HPF refers to high-power field (×400).

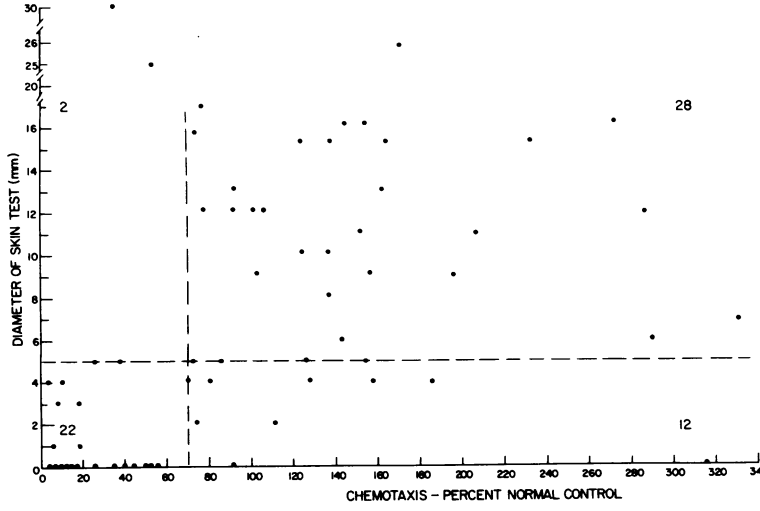

FIG. 1. *Relationship between chemotaxis and skin test reactivity in patient population studied. The diameter of skin test refers to the diameter of the largest skin test response in each patient.*

presence of anergy and inhibitor in each category is shown in Table 2. The miscellaneous category includes one patient with cholecystitis, one with gout, three with urinary tract infections, one with sideroblastic anemia, one with gonococcal arthritis, one with a pelvic abscess, one with appendicitis, one with seizures, one gun-shot wound, one patient with cellulitis, and one studied several days postpartum. Three patients in Table 1 were studied serially and results from times when both positive and negative skin tests were observed are included. Two of these patients were in the cirrhosis and liver disease category, and one was in the pulmonary category. In all three of these patients, the presence of chemotactic inhibitor was associated with skin test anergy.

Effect of anergic patient sera with chemotactic inhibitor on normal monocyte chemotaxis. Since delayed hypersensitivity is considered to be a mononuclear cell response, sera from four anergic patients with neutrophil chemotactic inhibitor were also tested with respect to inhibitory effect on monocyte chemotaxis. Representative results using four patients in two separate experiments are shown in Table 3. As can be seen, anergic-patient serum capable of inhibiting neutrophil chemotaxis was also capable of inhibiting monocyte chemotaxis. It should be noted that although chemotaxis was suppressed, random mobility did not appear to be affected.

Presence of cytotoxic antibody in serum from anergic patients. Since lymphocytotoxic antibody has the potential of being a cellular inhibitor, the presence of such an antibody in these patients was investigated. All sera tested were from anergic patients and contained serum chemotactic inhibitors. Only four of nine contained any lymphocytotoxic antibody (Table 4), indicating that this was not clearly associated with anergy. Furthermore, these results demonstrated that there was no relationship between cytotoxic antibody and chemotactic inhibitor, indicating that chemotactic inhibition was probably not mediated through a cytotoxic antibody in these patients.

TABLE 2. *Types of patients studied for presence of skin test anergy and serum chemotactic inhibitor*

Diagnosis	No. of anergic patients/total	No. of patients with inhibitor/ total
Cirrhosis and liver disease	8/13	6/13
Pulmonary disease	10/18	9/18
Septicemia and peritonitis	6/6	5/6
Fractures	0/4	0/4
Burns	1/2	1/2
Hodgkin's	0/1	0/1
Systemic lupus erythematosus	1/1	0/1
Mononucleosis	0/2	0/2
FUO[a]	3/4	1/4
Miscellaneous	5/13	2/13

[a] Fever of unknown origin.

TABLE 3. *Inhibition of monocyte chemotaxis in the presence of serum from four anergic patients*

Chemotactic factors[a]	Avg mononuclear (cells/5 OIF[b])	Actual chemotaxis (cells/5 OIF)	Inhibition (%)
10% Normal serum	27	11	0
MEM	16	0	
10% Ote. + 10% normal serum	17	1	91
10% Hol. + 10% normal serum	13	−4	100
10% Normal serum	85	61	0
MEM	24		
10% Nat. + 10% normal serum	16	−8	100
10% Arm. + 10% normal serum	37	13	55

[a] Ote, Hol., Nat., Arm., Anergic patient serum with chemotactic inhibitor.

[b] OIF, Oil immersion field.

T cells in anergic patients as determined by E binding. Since T cells are involved in the delayed-type hypersensitivity reaction, the possibility that T-cell suppression was related to the observed skin test anergy was investigated. The E-binding technique was utilized to determine the proportion of T cells in peripheral blood from these patients. Table 5 shows the results of this assay on normal controls, nonanergic hospitalized patients without chemotactic inhibitor, and anergic patients with or without chemotactic inhibitor. As can be seen, the average E binding for anergic patients was significantly lower than normal controls ($P < 0.01$) but not nonanergic hospitalized patients. A decrease in E binding in anergic patients was not a universal trait. Normal E-binding values were present in the anergic group, and depressed values also were recorded in the nonanergic patient population (Table 5). Patients showing lowest values for E binding among the nonanergic group (15 and 27%) carried diagnoses of burns and Hodgkin's disease, respectively. Overall, depressed T-cell proportions occurred more frequently among anergic pa-

TABLE 4. *Presence of lymphocytotoxic antibody in serum from patients with chemotactic inhibitor*

Patient serum studied	Chemotactic inhibition (%)	Target cell kill (%)						Avg kill (%)[a]
		Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	
Normal pool	0	10	10	10	10	10	10	10
Thom.	75	10	10	10	10	10	10	10
Val.	31	15	10	10	10	10	10	11
Oter.	95	70	50	30	70	40	80	57
Nat.	98	30	40	40	20	30	10	28
Bar.	72	10	10	10	10	10	10	10
Arm.	96	20	10	10	10	10	10	12
Yaz.	75	50	30	40	10	10	10	25
Yann.	98	10	10	10	10	10	10	10
Hol.	66	70	20	10	80	10	50	40

[a] Significant cytotoxic antibody was scored as being present when test serum showed 15% or greater average percentage of killing of target lymphocyte panel.

TABLE 5. *Relative proportions of peripheral blood T lymphocytes as determined by percent E binding among a group of normal controls, anergic and nonanergic patients*[a]

Normal controls	Nonanergic patient	Anergic patient
42	78	19[b]
77	70	55[b]
88	72	52[b]
71	77	73[b]
88	62	58
56	15	71
71	79	4[b]
52	40	58[b]
67	70	72[b]
71	75	47[b]
71	59	73[b]
63	27	54
59		60[b]
		70
		13
		47
		40
		53
Avg 67.4 ± 13.2	60.3 ± 21.4	51.0 ± 20.59

[a] Statistical analysis of E-binding suppression in anergic patients compared to: (i) normal controls = $P < 0.01$; (ii) nonanergic patients = $0.1 < P < 0.15$.

[b] Patient with chemotactic inhibitor.

tients. However, it was not clear whether this was a reflection of the type or severity of disease or related to skin test anergy.

Lymphocyte blast transformation in the presence of anergic serum. Previous studies by Brooks et al. (6) have demonstrated the presence of an IgG inhibitor of blast transformation in the serum of anergic patients with central nervous system tumors. To examine the possible presence of such an inhibitor in the anergic patients we accumulated, blast transformation experiments were performed by using normal lymphocytes in the presence of patient serum or IgG isolated by DEAE chromatography (Table 6). Part A was performed in the presence of 10% normal or patient serum. Part B was performed with a combination of both 10% fetal calf serum and 10% normal or patient serum to compensate for any lack of necessary components in the patient serum. Part C was performed in the presence of 10% normal human serum with the addition of the indicated quantities of DEAE-isolated normal or patient IgG fractions. All values are expressed as a stimulation index. Each control experiment was performed simultaneously with the patient experiment as indicated by the experiment number in Table 6. All anergic patient sera tested contained chemotactic inhibitor. These patients did not include individuals with diagnosed malignancies or systemic lupus erythematosus, where IgG inhibitors of blast transformation have been described (6; Cousar and Horwitz, Arthritis Rheum. **16**:539, 1973). No inhibitors of blast transformation were observed in any of the patient sera or IgG tested. In addition, partially purified chemotactic inhibitor isolated by DEAE (28) had no effect on mitogen-induced lymphocyte transformation. Parallel lymphocyte stimulation experiments using normal lymphocytes and pokeweed mitogen in the presence of serum from normal controls or anergic patients showed no significant difference in stimulation index.

DISCUSSION

Thirty-four of the 64 tests on 61 patients resulted in skin test responses of 5 mm or less. These patients were considered anergic. Of

TABLE 6. *Mitogen-induced lymphocyte blast transformation in the presence of normal, nonanergic, or anergic patient sera, or IgG fractions*

Culture medium	Expt no.	Stimulation index
Serum or serum fractions from normal controls		
A. 10% NHS[a]	1	103
	2	32
B. 10% NHS + 10% FCS[b]	1	63
	2	31
	3	25
	4	73
	5	115
C. 10% NHS + normal IgG		
1.2 mg/ml	1	157
0.8 mg/ml	2	35
Serum or serum fractions from nonanergic patients without inhibitor		
B. 10% Patient serum + 10% FCS	2	30
	4	68
	5	112
Serum or serum fractions from anergic patients with inhibitor		
A. 10% Patient serum	1	72
	2	52
B. 10% Patient serum + 10% FCS	1	65
	2	50
	3	31
	4	100
	5	106, 126
C. 10% NHS + IgG (patient)		
1.2 mg/ml	1	202
1.2 mg/ml	2	43

[a] NHS, Normal human serum.
[b] FCS, Fetal calf serum.

these 34 individuals, 65% demonstrated serum chemotactic inhibitors. The highly significant association of anergy and inhibitor ($P < 0.0005$) in these patients indicates that chemotactic inhibitor and suppression of skin test reactivity are closely related. Only two patients with chemotactic inhibitor were skin test positive. One of these patients had a lymphoproliferative disorder and gave a skin test response 30 mm in diameter. This is somewhat unusual and may have been related to the disorder. The other skin test-positive patient with chemotactic inhibitor was diagnosed as having gonococcal arthritis. This patient demonstrated a very high skin test response and had inhibitor at the 60% level. No clear explanation is yet available for these exceptions to the general pattern of high-degree association between chemotactic inhibitor and anergy. The remaining 22 patients (92%) with chemotactic inhibitor were anergic. Of these 22, 13 were totally negative in their skin test responses. Of the 40 patients without inhibitor, 28 were clearly skin test positive. Twelve patients fell into a category of anergy without chemotactic inhibitor. Although these patients were declared anergic by our initial criteria, many showed borderline responses and only two in this category were totally negative with respect to all of the skin test antigens. These data clearly indicate that there is a close association between chemotactic inhibitors and anergy in these patients.

Anergy itself was not peculiar to any particular disease, as demonstrated in Table 2. In addition to those patients investigated here, anergy has been demonstrated by others in lymphoproliferative disorders (8, 33), various cancers (6, 16, 21, 29), systemic lupus erythematosus (14), tuberculosis (15, 18), cirrhosis (26), myocardial infarction (Grossman et al., J. Allergy Clin. Immunol. **51**:127), T-cell deficiency diseases (11), patients with leukocytosis (13; Van Epps et al., Clin. Res. **21**:509), and in immunosuppressed patients (19, 25, 28). It is obvious that anergy may be related to a wide variety of clinical disease states. Brooks et al. (6) have described an IgG inhibitor of blast transformation associated with skin test anergy in patients with intracranial tumors. In addition, Cousar and Horwitz (Arthritis Rheum. **16**:539) have found similar inhibitors in patients with systemic lupus erythematosus. Our data indicate that in the patient population here, anergic patients possessing serum chemotactic inhibitors do not have serum suppressors of mitogen-induced blast transformation. Furthermore, no clear association was documented between presence of cytotoxic antibody and the anergic state or depressed T-cell proportions.

Overall, the data presented indicate that the presence of chemotactic inhibitors in the sera from anergic patients represents a close clinical parallel. The exact relationship remains to be defined. Chemotactic inhibitors may be involved in some type of normal immunological suppression and regulation in the host during various disease states.

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