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H Kondo, ... , B S Rabin, G P Rodnan

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### Research Article

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# Cutaneous Antigen-Stimulating Lymphokine Production by Lymphocytes of Patients with Progressive Systemic Sclerosis (Scleroderma)

HIROBUMI KONDO, BRUCE S. RABIN, and GERALD P. RODNAN

*From the Division of Clinical Immunopathology, Department of Pathology, and Division of Rheumatology and Clinical Immunology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261*

**ABSTRACT** Cell-mediated immunity to skin extracts was studied by the macrophage migration inhibition test, lymphocyte transformation, and direct cytotoxicity to skin fibroblasts, in normal individuals and patients with progressive systemic sclerosis. The latter included 18 individuals with diffuse scleroderma and 12 with the CREST syndrome, a variant form of systemic sclerosis in which there is more limited involvement of the skin. Controls consisted of 13 patients with other connective tissue diseases and 16 normal individuals. Phosphate-buffered saline and 3 M KCl extracts of both normal and sclerodermatous skin were used as antigens. No evidence of lymphocyte reactivity was found by the lymphocyte transformation and direct cytotoxicity test procedures. However, the lymphocytes of patients with diffuse scleroderma did respond to extracts of both normal and sclerodermatous skin in the migration inhibition assay. 10 of 16 patients (62.5%) had migration indices below 2 SD of the normal range. 1 of 10 CREST patients and 1 of 13 patients with other connective tissue diseases showed similar reactivity.

Antisera specific for immunoglobulin-bearing lymphocytes (B lymphocytes) and T lymphocytes were used to characterize the lymphocytes found in skin biopsies of patients with diffuse scleroderma. T lymphocytes made up the majority of lymphocytes in the skin infiltrates.

These findings suggest that lymphocytes sensitized to skin extracts are present in patients with diffuse scleroderma. The cell-mediated immune reaction to skin antigens may be a factor in the pathogenesis of diffuse scleroderma.

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## INTRODUCTION

Progressive systemic sclerosis (PSS,<sup>1</sup> scleroderma) is a generalized connective tissue disorder characterized by inflammatory, fibrotic, and degenerative tissue changes. Prominent vascular lesions are commonly found in the skin, synovium, and internal organs (1). The etiology of PSS is unknown. However, the high incidence of autoantibodies (2-4), the frequent association with Sjögren's syndrome (5, 6), and Hashimoto's thyroiditis, and the clinical overlap with systemic lupus erythematosus (5, 7, 8) suggest that immunologic abnormalities may be involved in its pathogenesis.

The role of antitissue antibodies in patients with PSS remains unknown (2, 9). The only immunoglobulin deposition found to date in the tissue of patients with PSS occurs in the glomeruli and the intralobular arteries of the scleroderma kidney (10). Lymphocyte accumulation within tissue of patients with PSS is common (11-13).

In delayed hypersensitivity skin tests and with *in vitro* response of lymphocytes to phytohemagglutinin as an indicator of cell-mediated immunity, patients with PSS responded normally (14). Circulating lymphocytes in patients with PSS have been found to be cytotoxic to cultures of fibroblasts, epithelial, and muscle cells (15). In addition the lymphocytes of patients with PSS release leukocyte migration inhibition factor (MIF) when cultured with autologous lymphocytes or with human liver microsomes or mitochondria (16).

<sup>1</sup>Abbreviations used in this paper: CREST, a form of PSS with calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, and telangiectasia; MIF, migration-inhibitory factor; PBS, phosphate-buffered saline; PSS, progressive systemic sclerosis.

The potential role of circulating lymphocytes as factors in the pathogenesis of PSS was studied. Lymphocyte transformation, the release of macrophage inhibition factor in response to skin extracts from patients with PSS and normal skin, and direct lymphocyte cytotoxicity to skin fibroblasts were evaluated.

## METHODS

**Patients.** 30 patients who met criteria for the diagnosis of PSS (scleroderma) were studied (1). There were 23 women and 7 men who ranged from 18 to 69 yr in age. The patients had been diagnosed from 7 to 264 mo before testing. These individuals all had abnormal thickening and tightness of the skin (scleroderma) and exhibited Raynaud's phenomenon. Esophageal dysfunction and pulmonary fibrosis and/or diminished pulmonary diffusing capacity were also present. In 18 there was diffuse scleroderma with involvement of the skin of the proximal portions of the extremities and the trunk, as well as of the fingers, hands, and face (classical scleroderma). 12 of the patients met the criteria of CREST syndrome, as previously defined (17, 18). In this form of PSS, there is prominence of calcinosis, Raynaud's phenomenon, esophageal dysfunction, limited involvement of the skin (often confined to the fingers) (sclerodactyly) and face, and telangiectasia. The individuals with the CREST syndrome have a relatively benign and protracted course of illness before the appearance of distinctive internal manifestations.

Controls consisted of nine patients with rheumatoid arthritis, three with systemic lupus erythematosus (each fulfilling diagnostic criteria of the American Rheumatism Association), one patient with mixed connective tissue disease (19, 20) and 16 normal individuals.

**Antigen extracts.** Skin (surgical specimens) was collected from normal individuals and from the forearm and legs of patients with PSS. Subcutaneous fat and connective tissue were removed from the skin. Normal liver and kidney were obtained at autopsy. Each tissue was minced with scissors and homogenized in a Sorvall Omni-mixer (DuPont Instruments, Sorvall Operations, Newtown, Conn.) at 0°C in phosphate-buffered saline (PBS), pH 7.2. 4 ml of PBS were added to each gram of tissue. After extensive homogenization, the extract was stirred at 4°C for 24 h and the homogenate centrifuged at 69,000 g for 30 min. Protein determination by the Lowry et al. method (21) was performed on the supernatant. The extracts of normal and PSS skin were adjusted to a concentration of 2 mg protein/ml. Kidney was 3 mg and liver 2.5 mg protein/ml. The pellet obtained after centrifugation was extracted in 3 M KCl (22). After centrifugation of the KCl extract, the supernatant was dialyzed against PBS for 72 h. The concentrations of both the KCl extracts were adjusted to 0.5 mg/ml. All of the extracts were sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.). Tissue extracts prepared from different patients gave the same results when used in the MIF assay.

**Lymphocyte separation.** Whole blood was collected into preservative-free heparin (1,000 U heparin/30 ml blood). The mononuclear cells were collected by Hypaque-Ficoll centrifugation by previously published methods (23). The lymphocytes were washed three times in tissue culture medium 199 (Grand Island Biological Co., Inc., Grand Island, N. Y.) containing 40 mM Hepes buffer, penicillin, and streptomycin.

**Migration inhibition test.** 25–30 ml of sterile mineral oil was injected into the peritoneal cavity of normal guinea

pigs. 72 h later, the animals were sacrificed and 100 ml of sterile Hanks' balanced salt solution injected into the peritoneal cavity. The fluid was collected and centrifuged at 264 g for 8 min. After three washes in cold balanced salt solution, the cell pellets were suspended in 10 vol of cold medium 199. The macrophages were counted and lymphocytes added so that 15% of the total cell suspension consisted of lymphocytes obtained from the patient. Capillary tubes were filled with the cell suspension, centrifuged, and cut at the cell-fluid interface. The capillary tube containing the cells was placed in Lab-Tek chamber slides (no. 4804, Lab-Tek Products, Div. Miles Laboratories, Inc., Naperville, Ill.) which were divided into eight separate chambers. To each of the chambers 0.4 ml of medium 199 containing 15% heat-inactivated fetal calf serum was added. A 1:10 final dilution of the various skin antigens was included in the medium of some chambers while the control chambers did not receive antigen. An additional control included with each experiment was macrophages without added lymphocytes.

The chambers were incubated for 18–24 h at 37°C. The area of migration was determined by projecting and tracing the image of the cellular migration within each chamber on a sheet of paper. The images were cut out and weighed. The migration index was calculated by dividing the average area of migration with antigen by the average area of migration without antigen and multiplying by 100.

All patients were tested in triplicate and the results used only if the SD was less than 15% of the mean. Migration inhibition was considered to be positive when it was less than 2 SD of the average migration inhibition of the 16 normal controls. Due to variable amounts of macrophages harvested from the guinea pigs, skin antigens were not used for each patient.

**Lymphocyte transformation.** Hypaque-Ficoll-purified mononuclear cells in a concentration of  $2 \times 10^5$  or  $1 \times 10^6$ /ml of medium 199 supplemented with 15% normal human AB plasma were added to 12 × 75-mm plastic culture tubes. To 1 ml of the lymphocytes, 0.1 ml of antigen extracts diluted 1:10, 1:100, or 1:1,000 in medium 199 were added. The cultures were incubated for 5 days at 37°C. Tritiated thymidine (specific activity 6.7 Ci/mM) was added 6–18 h before the termination of the culture. The cultures were harvested onto glass fiber filter paper and counted in a liquid scintillation counter. Each mononuclear cell population was run in triplicate at each antigen dilution. The results were calculated and the stimulation index was determined by dividing the counts per minute in the stimulated cultures by the counts per minute in cultures with no added antigen. To ascertain that the lymphocytes from the patients were capable of responding in vitro, each patient's lymphocytes were also incubated with phytohemagglutinin for 3 days and the stimulation index was calculated. In addition, lymphocytes from purified protein derivative-sensitive patients were incubated with purified protein derivative antigen or phytohemagglutinin, both in the presence and absence of skin antigen. The stimulation indices of the purified protein derivative and phytohemagglutinin cultures were the same regardless of the presence of the skin antigen. Thus, the skin antigen did not inhibit the proliferation of stimulated lymphocytes.

**Lymphocyte cytotoxicity studies.** Fibroblasts from the skin of normal individuals and patients with PSS were grown in tissue culture medium 199 containing 35 mM Hepes buffer and sodium bicarbonate and supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin, and nystatin. The fibroblasts were maintained in Falcon tissue culture flasks (3024 Falcon Plastics, Div. of BioQuest,

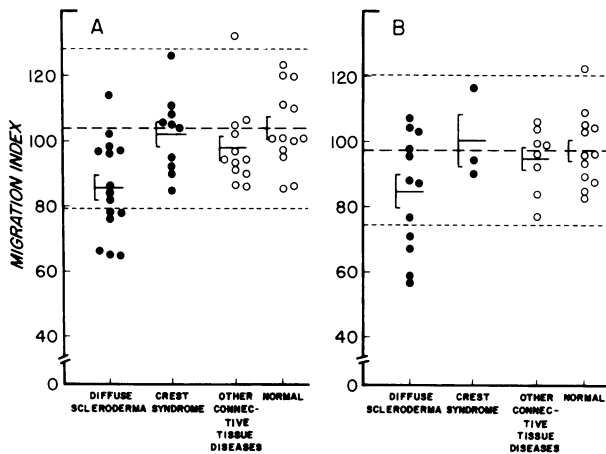


FIGURE 1 Migration indices of macrophages resulting from the incubation of lymphocytes of patients listed on the abscissa with normal skin extract in pH 7.2 PBS (A) and normal skin extract in 3 M KCl (B). The interrupted horizontal lines represent the mean and 2 SD for the normal controls. The bracketed horizontal lines are the mean and SE for each group.

Oxnard, Calif.). For cytotoxicity testing the monolayer was trypsinized, the cells suspended in medium 199, and  $10^5$  cells in 1 ml of tissue culture medium were added to each well of a Linbro tissue culture plate (Linbro Chemical Co., New Haven, Conn., no. FB-16-24TC). The cells in each well were labeled with 5–10  $\mu$ Ci of  $^{51}\text{Cr}$ . The free  $^{51}\text{Cr}$  was then washed from the cells and 1 ml of medium 199 containing  $2 \times 10^6$  lymphocyte added to each well.

The cells were incubated at 37°C for 24–72 h. The supernatants were then removed from each well and the wells washed once with 1 ml of medium 199. To each well was added 1 ml of 2% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) and after 24 h the Triton was removed and the wells were washed once with 1 ml of water. The percent cytotoxicity was calculated by dividing the amount of

TABLE I  
Lymphocyte Transformation by Skin Extracts

Antigen	Diffuse scleroderma	CREST syndrome	Normal controls	P
Normal skin in PBS	$1.58 \pm 0.17$ (18)	$1.78 \pm 0.19^*$ (12)	$1.21 \pm 0.15$ (11)	$< 0.025$
Scleroderma skin in PBS	$1.88 \pm 0.09$ (18)	$1.90 \pm 0.23$ (12)	$1.55 \pm 0.17$ (11)	NS
Normal skin in KCl	$1.18 \pm 0.09$ (12)	$1.42 \pm 0.26$ (8)	$1.11 \pm 0.14$ (5)	NS
Scleroderma skin in KCl	$1.31 \pm 0.15$ (7)	$1.50 \pm 0.30$ (8)	$1.09 \pm 0.21$ (5)	NS

Values are stimulation index, expressed as means  $\pm$  SE. P is by Student's *t*-test. Numbers of tests are in parentheses.

\* Significant difference from normal control.

radioactivity released into the wells cultured with lymphocytes by the maximum release. The maximum release was calculated by adding together the counts obtained from the supernatant and the counts obtained in the Triton solution. The final result was multiplied by 100. All tests were run in triplicate.

**Tissue immunofluorescence.** Punch biopsies of skin were taken from the forearm of patients with diffuse scleroderma at the area of demarcation between involved and normal skin or from involved skin. The tissue was embedded in O.C.T. compound (Lab-Tek Products, Naperville, Ill.) and frozen in a cryostat. Sections were cut at 4  $\mu$ m and fixed in cold acetone for 2 min. The tissue was then covered with fluorescein-labeled antisera to human immunoglobulins (Meloy Laboratories, Inc., Springfield, Va.) monospecific, for the heavy chains of IgG, IgA, and IgM (24). After 30 min the tissue sections were washed three times for 10 min with PBS and mounted in PBS:glycerol (90:10) for viewing in a fluorescence microscope. T lymphocytes were identified with a specific anti-T cell serum. Full characterization of this serum has been reported (24), as has its use in identifying T lymphocytes in tissue (25).

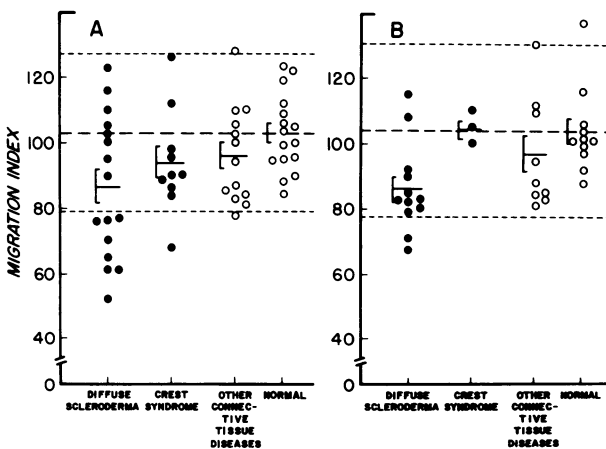


FIGURE 2 Migration indices of macrophages resulting from the incubation of lymphocytes of patients listed on the abscissa with PSS skin extract in pH 7.2 PBS (A), and PSS skin extract in 3 M KCl (B). The interrupted horizontal lines represent the mean and 2 SD for the normal controls. The bracketed horizontal lines are the mean and SE for each group.

## RESULTS

The results of the migration inhibition tests are shown in Figs. 1 and 2. The mean migration index for the peripheral blood lymphocytes of the patients with diffuse scleroderma was significantly less than the migration of the cells of normal individuals, with a *P* value  $< 0.005$  to all antigens except the normal skin extract in KCl. The *P* value was  $< 0.05$  with this antigen. Patients with the CREST syndrome did not differ significantly from normal controls. Thus, when the data for the diffuse scleroderma and CREST syndrome patients were pooled, the significant differences from normal individuals diminished. Patients with connective tissue diseases other than PSS did not differ from normal.

When tested against a saline extract of normal or PSS skin, the lymphocytes of 6 of 15 (40%), and 8 of 16 (50%) patients with diffuse scleroderma produced migration indices below 2 SD of the normal range. The response to KCl extracts of normal or PSS tissue

TABLE II

*Incidence of Positive Lymphocyte Transformation Responses (Stimulation Index >2) after Stimulation of Lymphocyte Culture with Antigen*

Antigen	Lymphocyte source			P*
	Diffuse scleroderma	CREST syndrome	Normal controls	
	% positive			
Normal skin in PBS	22.2 (18)‡	33.3 (12)	9.1 (11)	NS
Scleroderma skin in PBS	38.9 (18)	33.3 (12)	9.1 (11)	NS
Normal skin in KCl	0 (12)	12.5 (8)	0 (5)	NS
Scleroderma skin in KCl	0 (7)	25.0 (8)	0 (5)	NS

\* Chi square analysis with Yates' correction and Fisher's exact test.

‡ Number of tests.

was less pronounced. The cells of a total of 10 patients with diffuse scleroderma (62.5%) produced migration indices below 2 SD of the normal range to either an extract of normal of PSS skin. Seven patients, including three patients positive to skin extracts, were tested against normal human liver and kidney saline extracts. Mean migration indices and standard errors were  $98.5 \pm 4.3$  and  $101.6 \pm 4.3$ , respectively. No patient had a migrating index below 87%.

18 patients with diffuse scleroderma, 12 patients with the CREST syndrome, and 11 normal controls were tested for lymphocyte transformation to extracts of normal and PSS skin (Table I). Only the patients with the CREST syndrome had stimulation indices slightly ( $P < 0.025$ ) different from the normal controls when tested against the normal skin extract in PBS. The percentages of patients having stimulation indices greater than two to each of the antigens are shown in Table II. These results were not significant, at a  $P$  value of less than 0.05. The lymphocytes were tested at final antigen dilutions ranging from 1:100 to 1:10,000. Every patient tested had a stimulation index to phytohemagglutinin over 40. No significant differences were found in the mean counts per minute for background ( $1,267 \pm 560$ ;  $1,573 \pm 573$ ;  $1,085 \pm 740$  cpm), phytohemagglutinin stimulation ( $71,394 \pm 37,698$ ;  $93,559 \pm 61,572$ ;  $61,870 \pm 40,303$ ), or skin antigen stimulation ( $2,091 \pm 625$ ;  $2,690 \pm 1,261$ ;  $1,819 \pm 819$ ) when the normal controls were compared with the diffuse scleroderma and CREST syndrome patients. Similarly, the mean counts per minute of those patients with positive MIF responses did not differ from the patients whose lymphocytes did not produce MIF.

The direct cytotoxicity results are given in Table III. No significant differences were found between the

TABLE III  
*Lymphocyte Cytotoxicity*

Incubation time	Normal fibroblasts		Scleroderma fibroblasts		P*
	PSS lymphocytes	Normal lymphocytes	PSS lymphocytes	Normal lymphocytes	
<i>h</i>	%	%	%	%	
72	$50.1 \pm 2.2$ (8)	$49.3 \pm 2.8$ (8)	$48.7 \pm 1.5$ (10)	$48.5 \pm 1.7$ (10)	NS
48	$36.1 \pm 2.7$ (5)	$37.3 \pm 5.2$ (5)	$35.7 \pm 2.1$ (6)	$35.8 \pm 2.8$ (6)	NS
24	22.1 (1)	21.5 (1)	$20.5 \pm 1.2$ (2)	$20.2 \pm 1.2$ (2)	NS

% Cytotoxicity = (cpm of released  $^{51}\text{Cr}$  in the supernatant/cpm of maximum release of  $^{51}\text{Cr}$ )  $\times 100$ . Number of tests is in parentheses.

\* By Student's  $t$ -test.

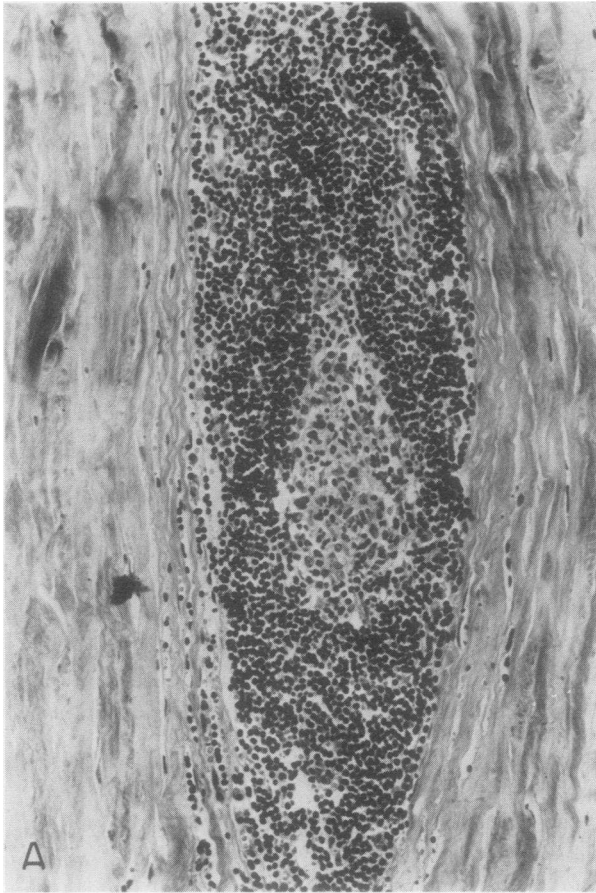
cells of patients with diffuse scleroderma and normal controls. This was true whether the fibroblasts were in early passages or skin cultures after repeated passages.

Skin biopsies were obtained from 17 patients with diffuse scleroderma. In 11 of the biopsies no lymphocytes bound fluorescein-labeled anti-immunoglobulin sera, yet eight of these had lymphocytes present on a section stained with hematoxylin and eosin

TABLE IV  
*Characterization of Lymphocytes in Skin Biopsies of Patients with Diffuse Scleroderma*

Hematoxylin and eosin stain	Staining of lymphocytes with antisera specific for:			
	IgG	IgA	IgM	T lymphocytes
ND	-	-	-	-
ND	-	-	-	-
±	-	-	-	-
ND	-	-	-	±
+	-	-	-	±
+	-	-	-	±
+	-	-	-	±
+	-	-	-	±
+	-	-	-	±
++	-	-	+	++
++	-	-	±	+
++	-	-	±	+
++	-	-	+	++
ND	+	±	±	-
+	+	+	-	±

ND, not done; -, no lymphocytes detected; ±, rare lymphocyte; +, scattered lymphocytes; ++, focal accumulations of lymphocytes.



**FIGURE 3** A. Photomicrograph of skin biopsy (deep dermis) from forearm of a 48-yr-old woman with PSS and diffuse scleroderma (H and E stain). Note band-like aggregation of lymphocytes and presence of a lymphoid follicle (160 $\times$ ).

(Table IV). Of the eight, seven were found to have only T lymphocytes in the tissue (Fig. 3).

## DISCUSSION

The production of MIF to an antigen indicates that lymphocytes are sensitized to that antigen (26–28). A migration inhibition assay showed that the lymphocytes of patients with PSS with diffuse scleroderma, but not those of patients with the CREST syndrome, are reactive to antigens found in normal and PSS skin but not in liver or kidney extracts. There was no difference in disease duration between the patients positive in the migration inhibition assay and those who failed to respond.

Most patients were studied at two different times. Of these, all but four had no change in reactivity. Three patients who were positive subsequently showed negative reactions in the MIF assay and one patient initially negative became positive. Several of the normal controls were studied at multiple time intervals and none of these ever became positive. In those

patients who showed a change of reactivity, no change in the activity of their disease was noted. However, long-term followup may be needed to ascertain the significance of the change in lymphocyte reactivity with the clinical course of disease.

HLA typing in this group of patients has previously been reported (29). There was no association between a particular HLA-A or B locus and the ability to respond with the production of MIF. In addition, preliminary results did not support an association between a particular HLA-D antigen and the ability to produce MIF (30).

Patients with PSS have a perivascular lymphocytic infiltrate in the dermis (12). It has recently been shown that substances derived from activated lymphocytes can stimulate fibroblasts to synthesize collagen (31). Thus, the sensitized lymphocytes may be a factor in the pathogenesis of PSS. Indeed, our finding of predominantly T lymphocytes in biopsies of skin from the patients studied (Table IV) is compatible with this interpretation. Due to the variability in the amount of lymphocyte infiltrate found on multiple biopsies of individual patients, no correlation with MIF production was possible.

In lymphocyte transformation, the mean stimulation index of the PSS patients was slightly higher than that of the controls. However, the only statistically significant result was obtained when the patients with the CREST syndrome were stimulated with a normal skin extract in PBS. All of the patients demonstrated normal reactivity to phytohemagglutinin both in the presence and absence of the skin extract. The dissociation between lymphocyte transformation and MIF results may indicate a selective alteration in a lymphocyte subpopulation (32), or the greater sensitivity of the migration inhibition test than the lymphocyte transformation assay. A similar dissociation of responsiveness has been demonstrated after the immunization of guinea pigs to collagen (33). The mean stimulation index of the patients who had a positive MIF response was not significantly different from those patients who were nonresponsive in the MIF assay.

Our studies did not reveal lymphocyte cytotoxicity to normal or PSS fibroblasts. Currie, et al. (15) demonstrated that patients with PSS had lymphocytes cytotoxic for fetal fibroblasts and epithelial and muscle cells. The difference between these findings and ours may be related to our use of adult tissue and not a cell like the L-cell line of fibroblasts, which are more sensitive to cell-mediated cytotoxicity (34).

Patients with diffuse scleroderma responded similarly to both normal and PSS skin extracts. Fleischmajer and Krol (35) have reported that a saline extract of scleroderma skin has the same concentration of hexose and hexosamine as well as of normal soluble protein. Some patients appeared to be reactive to glycoproteins

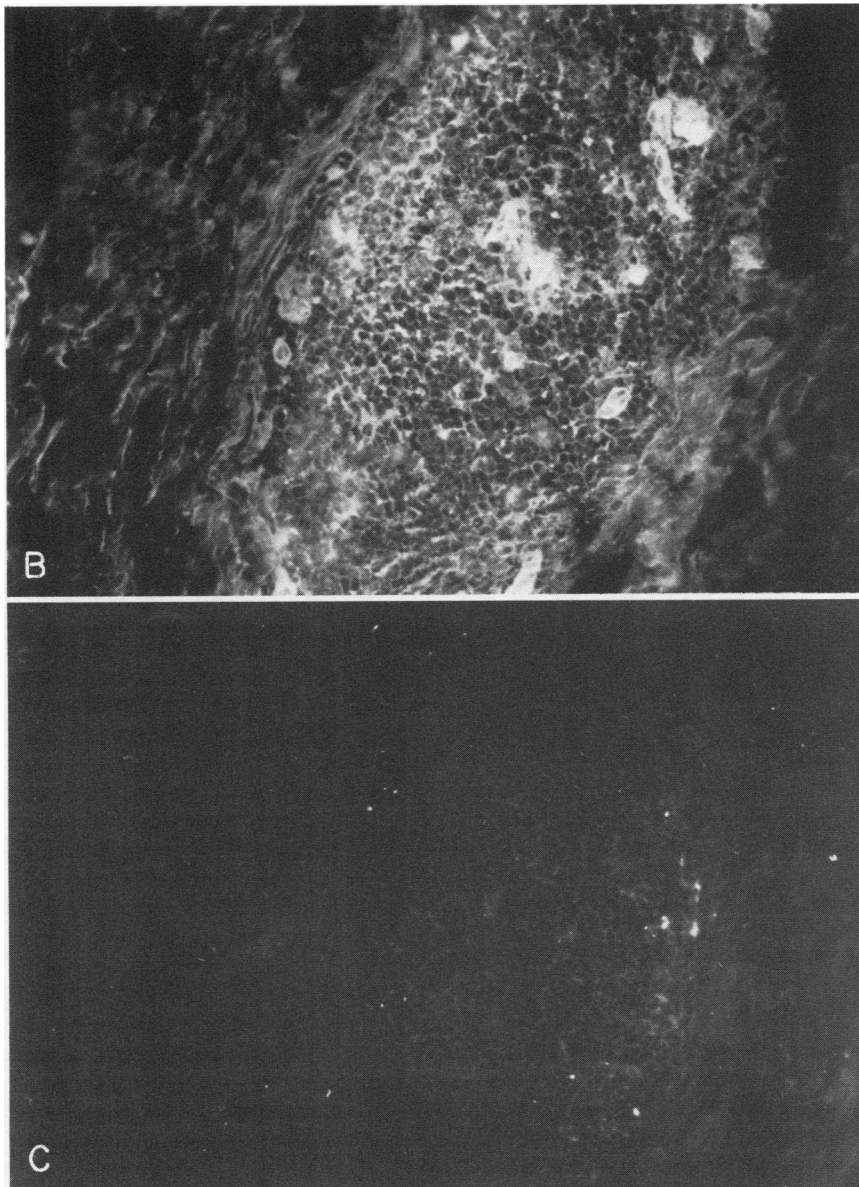


FIGURE 3 B. Same section of skin biopsy as in 3A, reacted with rabbit antiserum specific for T lymphocytes and fluorescein-labeled goat anti-rabbit IgG. All of the lymphocytes in the focal accumulation reacted with the antiserum (160 $\times$ ). C. Same biopsy as 3A, but reacted with fluorescein-labeled anti-human immunoglobulin serum. No lymphocytes were labeled (160 $\times$ ).

extracted in KCl. However, no difference was noted between PSS skin and normal skin.

These studies, concerning cell-mediated immunity to skin antigens in patients with PSS, indicate that lymphocytes in patients with diffuse scleroderma, unlike other connective tissue diseases with the exception of mixed connective tissue disease, are sensitive to skin extracts and may release MIF. The significance of this finding in the pathogenesis of PSS can only be speculative. However, the observation that lymphocyte mediators can stimulate collagen synthesis (31) is intriguing and suggests that a cell-mediated immune

response to skin antigens may be a factor in the pathogenesis of diffuse scleroderma.

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