Pathogenic mechanisms in pulmonary fibrosis: collagen-induced migration inhibition factor production and cytotoxicity mediated by lymphocytes.

T C Kravis, … , J D Fulmer, R G Crystal

J Clin Invest. 1976;58(5):1223-1232. https://doi.org/10.1172/JCI108576.

Research Article

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Pathogenic Mechanisms in Pulmonary Fibrosis

COLLAGEN-INDUCED MIGRATION INHIBITION FACTOR PRODUCTION AND CYTOTOXICITY MEDIATED BY LYMPHOCYTES

THOMAS C. KRAVIS, AFTAB AHMED, TIMOTHY E. BROWN, JACK D. FULMER, and RONALD G. CRYSTAL

From the Department of Clinical and Experimental Immunology, Naval Medical Research Institute, Bethesda, Maryland 20014, and the Pulmonary Branch, National Heart and Lung Institute, Bethesda, Maryland 20014

ABSTRACT The universal features of the histopathology of fibrotic lung disease are derangement of parenchymal collagen and infiltration of the parenchyma with chronic inflammatory cells. To determine if this cellular reaction might be associated with autoimmunity to a constituent of the alveolar interstitium, peripheral blood lymphocytes were exposed to human type I collagen in vitro and evaluated for the production of migration inhibition factor and cytotoxicity. Data from 18 patients with idiopathic pulmonary fibrosis, 8 patients with pulmonary fibrosis other than idiopathic pulmonary fibrosis, 12 patients with nonfibrotic lung disease, and 9 normals demonstrated that circulating lymphocytes from more than 94% of patients with fibrotic lung disease take part in processes where the recognition of collagen results in migration inhibition factor production and lysis of collagen-coated sheep red blood cells. These collagen-induced cell-mediated phenomena are obviated with human T-lymphocyte antiserum. Collagen-induced migration inhibition factor production and cytotoxicity were found in less than 20% of patients with nonfibrotic disease and were not found in normals. Qualitatively, there was no organ (lung, skin) or species (human, rabbit) collagen specificity in these assays, but human lung $\alpha 2$ chains were recognized more often than $\alpha 1$(I) chains. Circulating lymphocytes from patients with fibrotic disease are present in a normal T to B ratio. These lymphocytes did not incorporate [^{3}H]thymidine when exposed to collagen but did when exposed to T-cell mitogens. These in vitro observations suggest that circulating T-lymphocytes and lung collagen may be intimately associated in the pathogenesis of human fibrotic lung disease.

Send reprint requests to: Dr. Ronald G. Crystal, 6DO6, Building 10, National Institutes of Health, Bethesda, Md. 20014.

Dr. Kravis' present address is: Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California 92307. Dr. Kravis is the recipient of an American Thoracic Society Edward Livingstone Trudeau Fellowship.

Received for publication 13 April 1976 and in revised form 9 July 1976.

INTRODUCTION

The mechanisms leading to the development of pulmonary fibrosis are generally unknown. Even though a specific agent of lung injury can be identified in many cases, it is not understood how injury to the parenchyma leads to fibrosis, what prevents the reestablishment of normal lung structure, or why the fibrosis often progresses after the etiologic agent is removed (1).

The universal histologic features of all categories of pulmonary fibrosis are derangement of parenchymal collagen and infiltration with chronic inflammatory cells. As one approach to identifying pathogenic mechanisms common to all fibrotic lung disorders, we have reasoned that since parenchymal collagen appears deranged, immunologically competent cells in these patients might recognize collagen as "foreign." Collagen might then act as an antigen that maintains or causes progressive lung injury. As a first step in investigating this hypothesis, we used type I human lung collagen as an antigen to evaluate the in vitro reactive state of circulating lymphocytes from these patients.

METHODS

Study population

Patients were chosen from the clinical service of the Pulmonary Branch of the National Heart and Lung Institute. Most are part of a multidisciplinary study of interstitial lung diseases and their detailed clinical, roentgenographic, physiologic, and pathologic findings will be presented elsewhere. The study population was divided into four groups relatively homogeneous with respect to the diagnosis of fibrotic lung disease.

Group A—idiopathic pulmonary fibrosis (IPF).[1] 18 patients (53.5±3.7 yr;[2] 13 males and 5 females) were categorized as having IPF by the following criteria: a chronic, unremiting pulmonary disorder with an interstitial fibrotic pattern on chest film, reduced total lung capacity, reduced single breath diffusing capacity, reduced lung compliance, normal forced vital capacity (1 s)/forced vital capacity, and resting hypoxemia that worsened with exercise. 14 had open lung biopsies showing alveolar septal fibrosis and infiltration with chronic inflammatory cells and, intra-alveolar cellularity, but no granuloma. None of the biopsies showed refractile particles under polarized light microscopy. No patient had a history of chronic pulmonary infection, significant environmental exposure, symptoms suggestive of hypersensitivity lung disease, or left ventricular cardiac failure. None of the patients with IPF had circulating anti-nuclear antibodies; one had a positive rheumatoid factor; four had elevated serum IgA; but none had elevated serum IgG or IgM. No patients met the diagnostic criteria for a collagen-vascular disease. Eight were being treated with prednisone at the time of study.

Group B—pulmonary fibrosis other than IPF. Eight patients (48.4±4.3 years; five males and three females) had pulmonary fibrosis associated with a known etiologic agent or with a specific histopathologic process other than IPF. All had biopsy-proved parenchymal fibrosis except patients D. V. and P. K., who had all of the radiographic and physiologic criteria associated with group A, but also had strong histories of environmental exposure (D. V.) or viral pneumonia (P. K.). Both of the patients with chronic hypersensitivity pneumonitis (J. G. and R. B.) had precipitating antibodies in serum directed against thermophilic actinomycetes (*Micropolyspora faeni*).

Group C—nonfibrotic lung disease. 12 patients (42.3 ±4.7 yr; 8 males and 4 females) had pulmonary disorders not usually associated with fibrosis. Included were nine patients with diagnoses of acute or chronic bronchitis, asthma, chronic obstructive lung disease, or emphysema. In each case the history, roentgenographic and physiologic findings were consistent with criteria established for these diagnoses by the American Thoracic Society (2). The other three patients had acute pneumonia; one also had asthma. None of the patients in this group had physiologic findings usually associated with fibrotic lung disease. However, none underwent lung biopsy so the diagnosis of "no fibrosis" was not absolute.

Controls. Nine normal volunteers (35.0±4.1 yr; six males and three females) with no history or clinical evidence of pulmonary disease were used as controls.

[1] *Abbreviations used in this paper*: HTLA, rabbit anti-human T-lymphocyte-specific antiserum; IPF, idiopathic pulmonary fibrosis; MIF, migration inhibition factor; SRBC, sheep erythrocytes; VBS, veronal-buffered saline.

[2] All data are presented as means±SEM.

Preparation of protein antigens

Human fetal lung collagen was prepared from 16-wk-old fetuses as previously described (3). The human fetal lung was obtained after therapeutic abortion by curettage under regional anesthesia. The investigators in this study had no knowledge of the patients or involvement in the surgical procedure. After extraction, these collagen preparations were purified by multiple salt precipitations. Each preparation was more than 90% type I collagen; upon electrophoresis in sodium dodecyl sulfate acrylamide gels, $\alpha 1$, $\alpha 2$, β, and γ components could be identified. This material will be subsequently referred to as "collagen." $\alpha 1$(I) and $\alpha 2$ chains were purified from this collagen by carboxymethyl-cellulose chromatography; each $\alpha 1$(I) and $\alpha 2$ preparation was more than 95% pure (3).

Skin collagen was isolated from 10-wk-old rabbits fed 0.9% β-aminopropionitrile for 4 wk, as previously described (4). This collagen preparation was more than 90% pure and more than 95% type I collagen.

Human skin collagen was a gift of Dr. J. Harper; bovine serum albumin and transferrin were from Sigma Chemical Co., St. Louis, Mo; rabbit serum albumin was from Miles Laboratories, Inc., Elkhart, Ind.; and streptokinase-streptodornase was from Lederle Laboratories, Pearl River, N. Y.

The collagen preparations were dissolved in 1 M NaCl for 12 h at 4°C and centrifuged (1,000 *g*, 30 min). The supernatant was removed, dialyzed against 0.15 M NaCl, sterilized (0.22-μm Swinnex filter, Millipore Corp., Bedford, Mass.) and finally dialyzed against RPMI medium 1640 supplemented with L-glutamine (2 mM), Hepes buffer (25 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (final pH 7.4; Grand Island Biological Co., Grand Island, N. Y.) (This supplemented RPMI-1640 will be referred to as "medium"). Final collagen concentrations were determined by hydroxyproline analysis (4). The other protein antigens were dissolved in the same medium and sterilized.

Preparation of lymphocytes

Venous blood (50 ml) from each patient was drawn into a plastic syringe containing preservative-free sodium heparin (Medical Chemical Corp., Chicago, Ill.) and allowed to settle for 45 min with the syringe inverted. The leukocyte-rich plasma was removed and layered on Ficoll-Hypaque (specific gravity 1.078) (Pharmacia Fine Chemicals, Piscataway, N. J.) and centrifuged at 450 *g* for 45 min at 23°C. The mononuclear cells banding on top were washed twice with medium and resuspended to 3×10^7 cells/ml. An average preparation of cells contained more than 90% lymphocytes, with the remainder monocytes. This population of cells will be referred to as lymphocytes.

Assay for migration inhibition factor (MIF)

The indirect MIF assay used was a modification of the method of David and David (5, 6). Lymphocytes ($2-6 \times 10^6$ cells in 5–10 ml) were incubated in medium for 48–72 h in 75×100-mm tubes with various concentrations (1–50 μg/ml) of antigens, and the supernatant was collected after centrifugation. 50 μl of peritoneal exudative cells (5×10^7/ml), obtained from guinea pigs previously treated with intraperitoneal oil, were loaded into 75-mm capillary tubes sealed at one end. The capillary tubes were centrifuged (150 *g*, 10 min) and each tube was cut at the cell-fluid interface. The piece of tubing containing the peritoneal macrophages cells was affixed to the inside of a migration inhibition chamber (Míni Lab Co., Ville De Laval, Quebec) with sili-

cone grease, and the chambers (each containing two capillary tubes) were each sealed with a cover slip. Supernatant (0.5 ml) from the lymphocyte cultures was injected into the sealed chambers, and the chambers were incubated for 24–48 h at 37°C. After incubation, the outline of the migrated cells was traced with a projecting prism (Bausch & Lomb Inc., Scientific Optical Products Div., Rochester, N. Y.). The area of migration was determined by planimetry and the percent inhibition was calculated from the formula: % MIF = {1-[(average area of migration with antigen)/(average area of migration without antigen)]} × 100. Inhibition of migration of more than 20% was considered significant.

The direct MIF assay was performed similarly except the preincubation of lymphocytes with antigen was omitted. Instead, lymphocytes (2×10^7/ml) were mixed in equal volume with guinea pig peritoneal exudate cells (2×10^7/ml). A portion (50 μl) of this mixed suspension of lymphocytes and macrophages was introduced into each capillary tube and centrifuged (100 *g*, 10 min); the tubes were cut and affixed inside the migration inhibition chamber as described above. Antigen (1–50 μg/ml) in medium (0.5 ml) was introduced into the chamber and after a 24–48-h incubation at 37°C, the percent MIF was determined as described for the indirect assay. In both the direct and indirect assays, the area of migration for three controls (no antigen) and three assays with antigen were averaged before calculating the percent of MIF.

Lymphocyte-mediated cytotoxicity

Sheep red blood cells (SRBC) were kept as a 20% (vol/vol) suspension in Alsever's solution at 4°C. Portions of cells were washed three times in 0.9% saline (1,000 *g*, 5 min) and the final pellet was resuspended at 4% (vol/vol) in veronal-buffered saline, pH 7.4 (VBS). Equal volumes of 4% (vol/vol) SRBC and a tannic acid (4 μg/ml)-VBS solution were mixed and incubated at 23°C for 30 min. The SRBC were pelleted (1000 *g*, 5 min) and were washed three times with VBS. The tanned SRBC were then resuspended at 2% (vol/vol) in VBS. This suspension of cells was coated with collagen by mixing them with an equal volume of collagen (130 μg/ml) in VBS and incubating for 30 min at 23°C (7). Control tanned SRBC were coated similarly with rabbit serum diluted 1:150 in VBS. (The undiluted rabbit serum used throughout was previously absorbed with tanned SRBC to remove any anti-SRBC antibody). After incubation, the collagen and serum-coated SRBC were pelleted and washed three times with rabbit serum diluted 1:150, and the final pellet was resuspended at 1% (vol/vol) in diluted rabbit serum.

Freshly tanned and coated SRBC were pelleted and resuspended at 10% (vol/vol) in Hanks' balanced salt solution with bicarbonate buffer. The cells were then labeled with ^{51}Cr by adding 100–300 μCi of $Na_2[^{51}Cr]O_4$ (New England Nuclear, Boston, Mass.) in 0.9% saline to 3 ml of a 10% (vol/vol) suspension of cells and incubated at 37°C for 30 min. The coated, labeled SRBC were washed three times with saline (50 ml) and the final pellet was resuspended at 0.1–1.0% (vol/vol) in medium.

An appropriate dilution of tanned, labeled SRBC coated with collagen or rabbit serum was added to each well of a microtiter plate (Falcon Plastic, Div. of BioQuest, Oxnard, Calif.) in a volume of 0.1 ml. To this was added 0.1 ml of a suspension of lymphocytes. Each plate was agitated for 1 min, centrifuged at 180 *g* for 10 min, and then incubated at 37°C in 5% CO_2 and 95% air for 4 h. The plates were again agitated for 1 min and the cells were pelleted. Finally, 0.1 ml of the supernatant from each well was assayed for free ^{51}Cr in a gamma spectrometer (efficiency 60%). Each assay was done in triplicate. The cytotoxic activity of the lymphocytes was calculated as: percent net specific release of ^{51}Cr = [(mean cpm of lymphocyte mediated release) − (mean cpm of spontaneous release)] × 100/[(mean cpm of ^{51}Cr released from freeze-thawed SRBC) − (mean cpm of spontaneous release)] (6).

Lymphocyte transformation

The response of lymphocytes to the nonspecific mitogens phytohemagglutinin-P (Difco Laboratories, Detroit, Mich.), concanavalin-A (Calbiochem, San Diego, Calif.), and rabbit anti-human thymocyte globulin were tested in triplicate (6). 100 μl of lymphocytes in medium (2×10^6 cells/ml) containing 10% (vol/vol) of heat-inactivated (56°C, 30 min) fetal calf serum were cultured in a microtiter plate with 100 μl of an experimentally determined concentration of the appropriate mitogen. The cultures were incubated for 48 h (37°C, 5% CO_2 and 95% air), then pulsed with 20 μl of medium containing 1 μCi [^{3}H]thymidine (sp act 1.9 Ci/mmol, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) and reincubated for 18 h. Triplicate samples were harvested and the amount of [^{3}H]thymidine incorporated was determined by liquid scintillation spectrometry with an efficiency of 40%. Lymphocyte response was quantitated as a stimulation index: [(mean cpm with mitogen)/(mean cpm with medium)]. The response of lymphocytes to collagen was determined similarly except cells were also cultured for 72, 96, 120, and 144 h before harvesting.

Quantitation of T and B lymphocytes in peripheral blood

Lymphocytes were quantitated as T-cells by those forming erythrocyte (E) rosettes and as B-cells by those forming erythrocyte(E)-human 19S anti-Forssman(A)-mouse serum complement(C) rosettes as previously described (6).

Cytolysis of peripheral blood T-lymphocytes with anti-human T-cell-specific antiserum (HTLA)

Rabbit HTLA, previously shown to be highly specific for human T-lymphocytes, was prepared as described (6). Lymphocytes were incubated with antiserum (100 μl of antiserum lysed 3×10^7 T-lymphocytes) or normal rabbit γ-globulin at 37°C for 30 min. Rabbit serum complement (1:5 dilution) was then added and the incubation continued at 37°C for 15 min. The cells were pelleted and the remaining viable cells were isolated by banding on Ficoll-Hypaque. These cells were washed three times with medium and used in the MIF and cytotoxicity assays exactly as described above. The anti-HTLA-treated cells recovered from the second Ficoll-Hypaque procedure were 90% viable as tested by exclusion of trypan blue.

RESULTS

Production of MIF by peripheral lymphocytes from patients with pulmonary fibrosis. Collagen-induced production of MIF by peripheral blood lymphocytes was a common feature of patients with pulmonary fibrosis. When 25 μg/ml of collagen was used as the

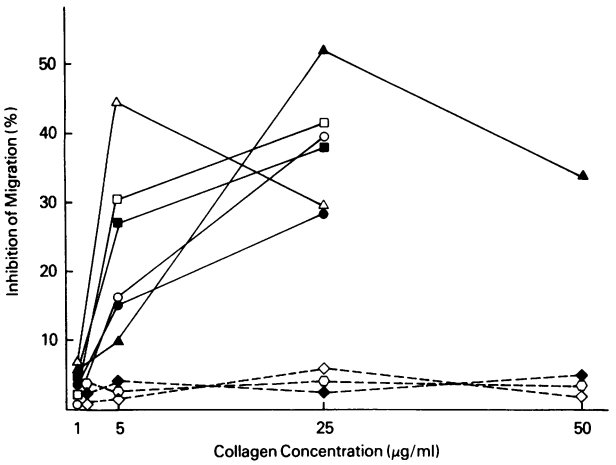

FIGURE 1 Relationship between the concentration of collagen and the production of MIF. Lymphocytes from six patients (△, ▲, □, ■, ○, ●) with pulmonary fibrosis were cultured (direct assay) with type I human lung collagen at concentrations ranging from 0 to 50 μg/ml. Three patients were from group A (△, ▲, ○) and three were from group B (□, ■, ●). Lymphocytes from three control subjects (◇, ◆, ◇) did not inhibit migration at each of the concentrations. When no collagen was used, inhibition of migration was less than 5% in all subjects.

antigen, lymphocytes from most patients with pulmonary fibrosis produced MIF, but those from normals did not (Fig. 1). For these reasons, 25 μg/ml of antigen was used for all subsequent experiments. 94% (17/18) of patients with IPF (group A) and 100% (8/8) of patients with pulmonary fibrosis other than IPF (group B) showed more than 20% inhibition of macrophage migration when their lymphocytes were exposed to human lung collagen in vitro (Table I, A and B). In striking contrast, only 17% (2/12) of patients with nonfibrotic lung disease (group C) and no normals (0/9) (group D) demonstrated 20% or more inhibition of macrophage migration under identical conditions (Table I, C and D). In patients with fibrotic disease (groups A and B), the average MIF activity (A, 30.6±2.3;[3] B, 38.4±1.5) was significantly higher than the control group (D, 4.6±0.8) ($P < 0.001$ in all comparisons).[4] The average MIF activity of the patients with nonfibrotic lung disease (C, 10.3±1.9) was also significantly higher than the control group ($P < 0.02$) but since the average MIF values for group C is well below the level of MIF activity considered positive (>20%), this difference probably has no meaning.

Patients whose lymphocytes produced MIF in response to collagen did so in serial determinations separated by several months. For example, in group A, patient A. H. showed 29, 30, and 44% and W. H., 12, 29, and 49%; in group B, R. B. showed 44, 44, and 28% and L. T., 33, 34, 30, and 63%. W. H. was the only individual tested whose percent MIF on ex-

[3] Mean±SEM.

[4] Student's two-tailed *t*-test.

TABLE I

Collagen-Induced Production of MIF by Peripheral Lymphocytes of Patients with Fibrotic Lung Disease

Group A: patients with IPF			Group B: patients with pulmonary fibrosis other than IPF				Group C: patients with nonfibrotic lung disease				Group D: normals		
Patient	Age/ Sex	MIF	Patient	Age/ Sex	Diagnosis	MIF	Patient	Age/ Sex	Diagnosis	MIF	Patient	Age/ Sex	MIF
		%				%				%			%
R.C.	66M	33.6±1.1	J.G.	47M	Chronic hypersensitivity pneumonitis	42.6±11.4	K.W.	29M	Asthma	7.4±2.7	A.A.	31M	−2.3±1.1
N.C.	27M	29.9±6.8					R.H.	30F	Asthma	2.4±1.0	T.K.	33M	4.6±2.9
A.M.	47M	34.2±6.9					R.C.	34M	Acute bronchitis	6.2±3.1	E.V.	50F	−5.8±1.8
G.S.	61F	39.6±10.2	C.W.	38M	Eosinophilic granuloma	41.3±2.9					S.K.	43F	−1.1±1.6
A.H.	26F	29.3±2.2					K.C.	34F	Chronic bronchitis	5.7±3.2	D.N.	20M	−3.0±2.1
W.H.	41M	29.0±3.0	D.V.	45M	Pneumoconiosis	33.7±4.5					B.L.	27M	9.2±3.3
A.R.	75M	21.3±2.7					J.C.	63M	Emphysema	9.5±2.3	P.L.	21M	3.1±2.0
B.T.	47F	24.9±2.4	M.C.	54F	Sarcoidosis	33.6±4.6	E.M.	63M	Chronic bronchitis, obstructive lung disease	21.4±4.1	B.O.	56M	5.9±3.2
A.D.	67M	28.6±3.2	O.D.	41M	Granulomatous lung disease	38.6±5.3					G.M.	34F	6.1±2.5
S.C.	43M	39.9±4.5											
E.B.	48M	23.2±5.1	P.K.	65M	Post-infectious fibrosis	39.5±5.7							
P.R.	31F	53.7±7.4					N.E.	29M	Asthma	5.8±2.1			
R.S.	52M	33.7±7.1	R.B.	65F	Chronic hypersensitivity pneumonitis	44.2±7.9	B.K.	36M	Acute pneumonia	20.0±4.8			
M.A.	78F	26.3±3.4					T.C.	70M	Chronic obstructive lung disease	1.5±3.6			
W.E.S.	66M	30.3±2.4											
W.L.S.	58M	37.6±5.5	L.T.	32F	Lymphangitic pulmonary myomatosis	33.7±4.6							
W.L.	67M	5.9±2.1											
J.S.	63M	29.9±3.4											
							S.S.	56F	Acute pneumonia	15.2±2.8			
							R.R.	22M	Asthma	15.9±3.4			
							J.N.	41F	Asthma, pneumonia	12.8±4.7			

Type I human lung collagen (25 μg/ml) was used for all assays.

TABLE II

Comparison of Collagen-Induced MIF with the Direct and Indirect MIF Assays

Patient	Group	Migration Inhibition	
		Direct assay	Indirect assay
		%	
A.D.	A	39.6±4.7	31.4±4.7
B.T.	A	41.4±5.3	36.9±7.8
W.H.	A	29.1±4.8	27.3±5.6
N.C.	A	56.9±6.4	44.9±6.6
J.S.	A	24.5±1.2	27.9±3.4
O.D.	B	31.2±4.7	38.5±5.7
A.A.	D	12.9±3.3	16.7±4.1
C.C.	D	15.1±4.9	12.3±3.9

25 μg/ml of type I human lung collagen was used in each incubation.

posure to collagen changed from negative to positive on serial testing. No patient had a positive collagen-induced MIF production that later reverted to negative. Collagen-induced MIF production assayed by the direct and indirect methods showed no differences attributable to the assay procedure (Table II) (8).

To further characterize the supernatant from the indirect MIF assay, lymphocytes from one patient with IPF (N.C.) and one control (A.A.) were incubated with human lung collagen, the cells pelleted, and the supernatants separated into three molecular weight fractions (Table III). In the patient with IPF, the antigen (collagen) was in the void volume (>100,000 daltons) while the MIF activity was in the included volume (<60,000 daltons). This is consistent with the known molecular weights of MIF (9) and human lung collagen (4). In the control, the collagen was still present in the void volume, but there was no MIF activity in any of the fractions tested (Table III). That MIF activity produced by IPF lymphocytes was contained in a fraction separate from the antigen obviates the possibility that the results of the MIF assay were secondary to a phenomena involving collagen chemotaxis (10).

Antigenic specificity for MIF production. The human lung collagen used was over 95% pure type I collagen and it was probably in the native, triple-helical form (or higher aggregates) when incubated in vitro in medium at 37°C. When this material was further purified into component α1(I) and α2 chains, it still induced production of MIF in most patients tested who had pulmonary fibrosis (Table IV). Although some patients in groups A and B did not respond to 25 μg of α1(I) chains (A. H., W. H., and D. V.), all patients in groups A and B who were tested responded to α2 chains. No patients tested in groups C or D produced MIF in response to α1(I) or α2 chains,

TABLE III

*Partial Purification of MIF Produced by Lymphocytes of a Patient with IPF in Response to Collagen**

Patient	Group	Fraction‡	Migration inhibition§	Hydroxyproline
			%	μg/ml
N.C.	A	I	18.7±4.1	166.
		II	12.1±3.0	27.
		III	49.6±6.7	12.
A.A.	D	I	19.6±2.9	151.
		II	12.4±1.8	56.
		III	9.2±3.4	<5.

* Lymphocytes (2×10^6/ml) were cultured for 72 h in the presence of human fetal lung collagen (25 μg/ml) in a total volume of 20 ml; the cells were pelleted (1,500 g, 10 min) and the supernate was concentrated to 2 ml with an Amicon UM-2 membrane (Amicon Corp., Scientific Systems Div., Lexington, Mass.).

‡ Sephadex G-100 (Pharmacia Fine Chemicals) (5 × 42 cm; 25 ml/h) chromatography of the concentrated supernatants. 5-ml fractions were collected and pooled into three fractions. Starting at the void volume (0 ml), fraction I = 0–75 ml; fraction II = 75–135 ml; and fraction III = 135–230 ml.

§ Each fraction was pooled, concentrated to 2 ml (Amicon UM-2 membrane), dialyzed against 0.15 M NaCl; and then dialyzed against RPMI-1640; and then used in the indirect MIF assay with guinea pig macrophages as indicator cells.

TABLE IV

α1(I) and α2 Human Collagen Chain-Induced Production of MIF by Peripheral Lymphocytes of Patients with Fibrotic Lung Disease

Patient	Group	MIF		
		Collagen*	α1(I) chains*	α2 chains*
			%	
A.H.	A	29.3±2.2	15.2±2.9	30.7±1.2
W.H.	A	29.0±3.0	15.9±6.2	26.2±2.1
C.W.	B	41.3±2.9	28.9±2.7	30.6±2.9
D.V.	B	33.7±4.5	15.6±1.1	28.5±4.2
P.K.	B	39.5±5.7	32.6±4.9	28.7±3.4
R.B.	B	44.2±7.9	26.8±1.9	37.1±6.8
L.T.	B	33.7±4.6	37.5±5.9	28.4±3.4
B.K.	C	20.0±4.8	1.2±1.1	1.1±1.1
T.C.	C	1.5±3.6	4.8±2.2	0.8±1.9
A.A.	D	−2.3±1.1	1.9±2.3	−7.6±5.4
T.K.	D	4.6±2.9	5.3±1.5	7.2±2.1
E.V.	D	−5.8±1.8	−2.9±1.6	−1.7±2.2
S.K.	D	−1.1±1.6	−1.4±1.1	−0.8±0.8
D.H.	D	−3.0±2.1	1.8±1.5	1.1±1.1
B.L.	D	9.2±3.3	4.6±2.2	2.5±2.0
P.L.	D	3.1±2.0	1.8±1.1	5.4±2.2
B.O.	D	5.9±3.2	3.8±1.9	5.7±3.2
G.M.	D	6.1±2.5	2.0±1.8	5.2±3.4

* 25 μg/ml of collagen, α1(I) chains, or α2 chains were used in each incubation.

including the one patient in group C (B. K.) who had a MIF value of 20.0±4.8% in response to collagen.

There seemed to be no difference (i.e., MIF positive or negative) whether the collagen presented to the lymphocytes was human lung collagen, human skin collagen, or rabbit skin collagen. Comparison of 25 μg/ml of bovine serum albumin, rabbit serum albumin, human transferrin, and collagen as antigens for lymphocytes of patients in groups A and B showed that only collagen resulted in production of MIF. Random checks of patients in group C and group D (normals) who did not produce MIF in response to collagen showed that their lymphocytes could produce MIF when exposed to streptokinase-streptodorinase in vitro.

Collagen-induced cell-mediated cytotoxicity. Preliminary experiments demonstrated that a 0.1% (vol/vol) suspension of collagen-coated SRBC allowed optimum specific release of ^{51}Cr when the labeled SRBC were incubated with peripheral blood lymphocytes of patients with pulmonary fibrosis. A ratio of lymphocytes to SRBC of 5:1 gave maximum percent net specific release of ^{51}Cr (Fig. 2) and this ratio was used in all subsequent experiments. Although all control lymphocytes (group D) gave a percent net specific release of ^{51}Cr from collagen-coated SRBC of less than 2%, all patients in groups A and B who were tested gave a net specific release of greater than 2% (Fig. 3). When human or rabbit collagen was used as the antigen, equivalent results were found.

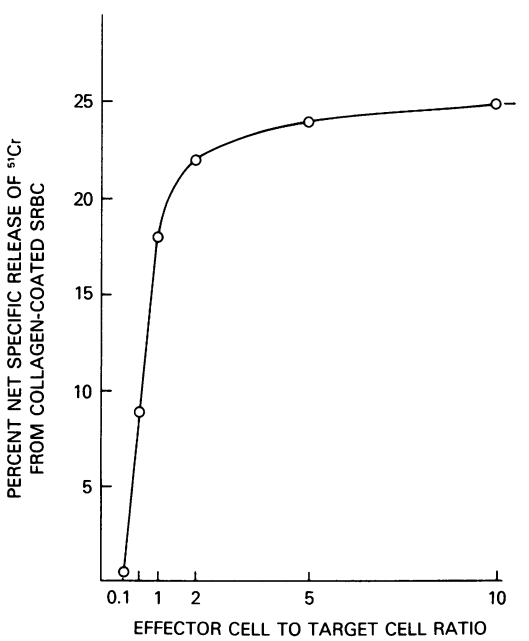

FIGURE 2 Release of ^{51}Cr from collagen-coated SRBC induced by lymphocytes from a patient with pulmonary fibrosis. Effector cell (lymphocyte) to target cell (collagen-coated SRBC) ratios of 0.1:1 to 10:1 were used.

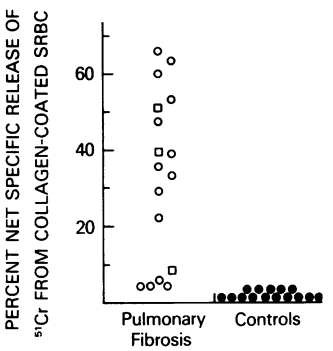

FIGURE 3 Collagen-induced cytolysis of SRBC by lymphocytes from patients with pulmonary fibrosis. Data from 14 patients with IPF (○), 3 patients with pulmonary fibrosis other than IPF (□), and 15 normal controls (●) are shown. In all cases, an effector cell to target cell ratio of 5:1 was used.

Identification of the T-lymphocyte as the cell mediating MIF production and cytotoxicity. When lymphocytes from patients in group A or B were pretreated with rabbit anti-human T-lymphocyte specific antiserum and rabbit complement before the MIF assay, there was no collagen-induced MIF production (Table V). In contrast, pretreatment of lymphocytes from the same patients with medium alone or medium plus normal rabbit γ-globulin and rabbit complement did not alter the induction of MIF production. In the studies with HTLA, the antiserum-sensitive cells were removed (by centrifugation with Ficoll-Hypaque) from the remaining lymphocytes before their incubation with collagen. This is important, since the antiserum-sensitive cells could still produce MIF when exposed to collagen. For example, when the lymphocytes of a patient with IPF (A. D., group A) was treated with antiserum and complement and then exposed to collagen (without removal of the antiserum-sensitive cells), MIF was still produced (39.6±4.7 before antiserum, 41.7±3.9 after antiserum). However, if the treatment with antiserum was followed by removal of the antiserum-sensitive cells (by centrifugation with Ficoll-Hypaque), no MIF was produced (15.4±5.2). Similar results were obtained with lymphocytes of patient N. C. (group A; 56.9±6.4 before, 46.8±4.6 after, 14.9±4.7 after + Ficoll-Hypaque). Thus, whenever the HTLA was used, it was necessary to reisolate the remaining lymphocytes with Ficoll-Hypaque banding before incubation with the antigen.

Pretreatment of lymphocytes with rabbit HTLA and rabbit complement completely alleviated collagen-induced cytotoxicity, while pretreatment with normal rabbit γ-globulin and rabbit complement

TABLE V

Identification of the T-Lymphocyte as the Cell Responsible for Collagen-Induced MIF Production and Cytotoxicity

Patient	Group	Collagen-induced MIF:* lymphocyte pretreatment			Collagen-induced cytoxicity:‡ lymphocyte pretreatment	
		Media	NRγG§ + complement	HTLA + complement	NRγG + complement	HTLA + complement
			%		%	
R.S.	A	33.7±7.1	28.5±4.2	4.1±1.9	52.7	4.1
N.C.	A	29.9±6.8	21.2±2.4	2.7±0.8	46.9	5.9
A.M.	A	34.2±6.9	28.4±4.1	3.3±1.1	62.7	5.9
G.S.	A	39.6±10.2	32.6±2.8	2.2±0.9	38.8	4.7
J.G.	B	42.6±11.4	37.4±5.6	1.5±1.2	38.8	4.2
A.A.	D	4.9±2.2	2.9±2.1	1.5±1.4	2.4	5.9

* All MIF assays were done with 25 μg/ml of type I human fetal lung collagen.

‡ All cytotoxicity assays were done with an effector cell to target cell ratio of 5:1. Data are presented as percent net specific release of ^{51}Cr from collagen-coated sheep red blood cells.

§ NRγG, normal rabbit γ-globulin.

allowed the collagen-induced cytotoxicity to be present.

As further evidence that the HTLA destroyed T-cells, the population of viable lymphocytes recovered after incubation with antiserum and complement and then reisolated on Ficoll-Hypaque would not respond to phytohemagglutinin-P or concanavalin A (data not shown).

Even though the lymphocytes (before treatment with anti T-lymphocyte antiserum) from patients in groups A and B incorporated [^{3}H]thymidine normally in response to the nonspecific mitogens phytohemagglutinin-P, concanavalin A, and rabbit anti-human thymocyte globulin, they did not incorporate [^{3}H]-thymidine when exposed to collagen in vitro (Fig. 4). The in vitro response of lymphocytes from patients in groups A and B to the nonspecific mitogens peaked on day 3, as did the normal controls. The response of lymphocytes to collagen was evaluated by culturing cells with various concentrations of collagen for 48, 72, 96, 120, and 144 h. These longer incubation periods still failed to demonstrate significant [^{3}H]thymidine incorporation.

The number of T-lymphocytes in the peripheral blood of patients with pulmonary fibrosis were within the normal range, as were the number of B-cells (Fig. 5).

DISCUSSION

The data presented here suggest that lymphocytes from patients with fibrotic lung disease are sensitized to type I collagen. In almost every case, exposure of peripheral blood lymphocytes from these patients to this collagen in vitro results in the production of MIF and specific cytolysis of collagen-coated cells. This phenomena are unusual in patients with nonfibrotic lung disease and are not found in normal individuals. The experiments with HTLA demonstrate that the T-lymphocytes are, in some fashion, involved in this process of recognition of collagen

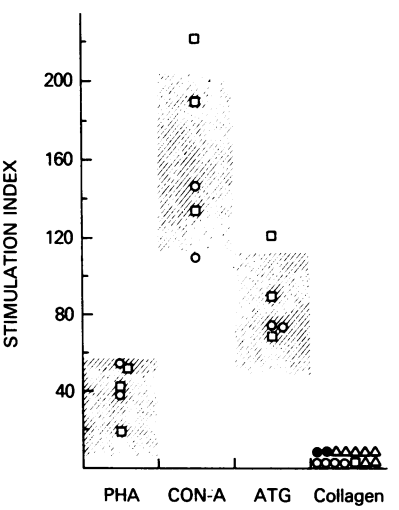

FIGURE 4 Reactivity of peripheral lymphocytes of patients with IPF (○), pulmonary fibrosis other than IPF (□), patients with nonfibrotic lung disease (△), and normal controls (●) to optimum concentrations of phytohemagglutinin (PHA), concanavalin-A (CON-A), anti-thymocyte globulin (ATG), and collagen. The stimulation index is the mean counts per minute of [^{3}H]thymidine incorporation in the presence of mitogen (or collagen) divided by the mean counts per minute of the media control. For the PHA, CON-A, and ATG data, the shaded areas show 95% confidence limits for normal controls in our laboratory (6).

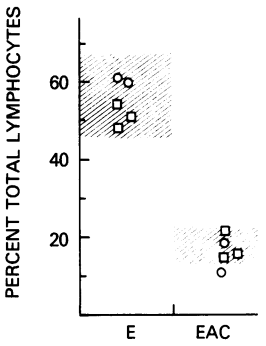

FIGURE 5 Quantitation of T-lymphocytes (E rosettes) and B-lymphocytes (EAC rosettes) in the peripheral blood of patients with IPF (○) and patients with pulmonary fibrosis other than IPF (□). The shaded areas show the 95% confidence limits for normal controls in our laboratory (6).

as antigenic and the subsequent production of MIF and cytotoxicity.

Collagen as an antigen. This is not the first example of a clinical disorder in which there is in vitro evidence of immune reactivity to collagen, the most abundant protein in the body. Antibodies against collagen have been found in the sera of patients with rheumatoid arthritis, emphysema, and selective IgA deficiency (11–14). Anti-collagen antibodies have also been found in the synovial fluid of patients with rheumatoid arthritis and traumatic synovitis (15), and collagen-anticollagen immune complexes have been found in synovial fluid cells of patients with rheumatoid arthritis (16).

While most clinical examples of immunity to collagen have involved the humoral immune system, animal experiments have demonstrated that under the proper circumstances, immunization with collagen incites not only circulating collagen antibodies (17–21), but cell-mediated immunity as well (22–24). Although most of these experimental studies capitalized on species differences in collagen antigenicity to invoke an immune response, Senyk and Michaeli have experimentally induced cell-mediated immune phenomena to homologous collagen in the guinea pig (25).

The human type I collagen was probably in the native, triple-helical state under the conditions of the lymphocyte incubations (although this is difficult to prove, particularly in the cytotoxicity assay). Thus, it is most likely that the triple-helical form of collagen is the antigen to which the lymphocytes respond in these assays. In addition, the studies with purified α1(I) and α2 chains suggest that denatured collagen is also recognized as antigenic by the lymphocytes of these patients and that α2 chains probably have more antigenic determinants than do α1(I) chains (17, 18, 21). However, the recognition of collagen by lymphocytes from patients with fibrotic lung disease is not very specific; they do not distinguish between type I collagen of different species (human, rabbit) or different organs (lung, skin). Since prior studies have shown these type I collagens to be very similar (3, 4, 26–29), this cross-reactivity is not surprising.

This lack of specificity demands that if these collagen-induced cell-mediated phenomena are important in vivo, they must occur on a local level, since there is no evidence that the typical patient with fibrotic lung disease has pathologic processes elsewhere. Thus, there must be local lung factors promoting the recognition of collagen as antigenic and/or allowing the collagen-activated cell-mediated immune system to respond. Possible mechanisms that would result in collagen-induced immune reactions in lung but not other tissues include: (*a*) the structure of lung collagen is altered (e.g., denatured, fragmented); (*b*) lung collagen is normal but is presented to the local cellular immune system in an abnormal way (e.g., alteration of normal collagen-proteoglycan interaction); or (*c*) lung collagen is unchanged but the local cell-mediated immune system is somehow abnormal (e.g., enhanced immune reactivity, alteration in normal macrophage-lymphocyte interactions (30), or loss of local T-lymphocyte suppressor function [31]). However, if the cell-mediated immune system is abnormal, it is not the absolute number or reactivity of T-lymphocytes alone, since in our patients these cells were present in normal amounts, reacted to T-mitogens normally, and did not spontaneously transform. It is also possible that the T-lymphocytes reacting to collagen are a special subset of the lymphocyte population present in patients with pulmonary fibrosis. Interestingly, even though the lymphocytes from patients with fibrotic disease produced lymphokines when exposed to collagen in vitro, collagen did not induce transformation of these cells. Thus, collagen-induced cell-mediated immunity in fibrotic lung disease may be another example of the possible separation of lymphocyte transformation and lymphokine production (32).

These experiments do not suggest that type I collagen is the only antigen that could cause these phenomena in fibrotic lung disease. The normal human lung parenchyma is known to be composed of at least two types of collagen (types I and III) (26), in addition to smaller amounts of the less well described type IV (basement membrane) collagen (33). We have not, as yet, examined the antigenicity of types III and IV collagens in these patients. It is also possible that other connective tissue components from the "deranged" interstitium might act as antigens.

Similar phenomena might also be found in other fibrotic processes (e.g., wound healing, fibrosis of other parenchymal organs), in conditions associated with collagen destruction (e.g., postpartum uterus, extensive burns) or in other disorders associated with connective tissue abnormalities (e.g., scleroderma) (34). The rarity of collagen-induced MIF production in group C suggests that collagen does not usually invoke cell-mediated immune phenomena in nonfibrotic lung disease, but a larger group will have to be examined to validate this finding.

In vivo significance of these in vitro assays. Although we used guinea pig peritoneal macrophages as the target cell for human lymphocyte MIF production, there is evidence that the human alveolar macrophage responds to human lymphocyte MIF (35, 36). Thus, MIF production could be responsible for the large accumulation of macrophages in the lung parenchyma of patients with fibrotic lung disease. Stimulation of these macrophages could result in the secretion of numerous destructive enzymes, including collagenase, an enzyme specific for collagen (37, 38). There is also evidence that stimulation of lymphocytes could result in macrophage proliferation (39) and that macrophages can ingest collagen-anticollagen immune complexes (40). Thus, the recognition of collagen as antigenic by cells in the lung parenchyma theoretically could foment numerous macrophage-lymphocyte-collagen interactions important in the continued tissue destruction in this disease.

As in the in vitro lysis of collagen-coated red blood cells, collagen-activated lymphocytes might utilize lung fibroblasts (or any lung collagen-synthesizing cell) as target cells. This is suggested by data demonstrating that: (*a*) collagen is bound to membranes of fibroblasts (41); (*b*) human lung fibroblasts synthesize type I collagen (28); and (*c*) antibodies to collagen are cytotoxic to fibroblasts in culture (42–44).

In addition to the cell-mediated phenomena investigated in IPF, studies of the lavage fluid from these patients have demonstrated that the epithelial surfaces of the lower respiratory tracts of these patients have more polymorphonuclear leukocytes and eosinophils than normal (45). Probably, a number of as yet uninvestigated cell-cell interactions are also important in the pathogenesis of fibrotic disease, of which collagen-induced MIF production and cytotoxicity are just two.

It is possible, of course, that the cell-mediated reactivity to collagen found in patients with fibrotic disease is secondary to chronic lung injury rather than being of primary pathogenic importance in causing the injury. Even so, these phenomena may be significant in further extending the already deranged alveolar interstitium.

ACKNOWLEDGMENTS

The authors wish to thank K. Bradley, who purified the human lung collagen, and K. Cook, J. Barchers, and N. Wyne for their editorial assistance.

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