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R L Johnson, M Ziff

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**Research Article**

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# Lymphokine Stimulation of Collagen Accumulation

ROBERT LEE JOHNSON and MORRIS ZIFF

*From the Rheumatic Diseases Unit, Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas 75235*

ABSTRACT Lymphokine-rich supernates from normal human peripheral blood mononuclear cells, stimulated by the mitogen phytohemagglutinin, have been shown to cause enhanced collagen accumulation by human embryonic lung fibroblasts (WI-38), as measured by hydroxyproline content of fibroblast monolayers, [$^{14}$C]proline incorporation into soluble collagen and collagenase release of radioactivity in supernates and monolayers of cultures incubated with [$^{14}$C]proline. This fibroblast-stimulating activity, demonstrable by suitable dilutions of the supernates, coexisted with a number of other lymphokine activities such as lymphotoxin, proliferation inhibitory factor, and cloning inhibitory factor, which tend to reduce the numbers or function of fibroblasts. The increased content of collagen appeared to be the product of selected surviving and responding fibroblasts. The factor causing this increased collagen accumulation was nondialyzable and stable at $-70$°C. It represents the first described lymphoid cell-derived activity capable of enhancing collagen accumulation. Fibroblast-stimulating activity may be implicated in the abnormal fibrosis seen in association with chronic inflammation in a variety of disease states. It may have special relevance to progressive systemic sclerosis.

## INTRODUCTION

Chronic inflammatory infiltrates containing predominantly monocytes and lymphocytes are a characteristic feature of the cellular immune response (1). Such infiltrates are also commonly seen in association with the fibrosis consequent to chronic inflammation. In progressive systemic sclerosis (PSS),[1] the association of skin and visceral fibrosis with mononuclear cell infiltration has been observed, although not emphasized (2–10).

Vascular changes are regularly seen in PSS, Raynaud's phenomenon is very commonly present (11), and the vascular capillary bed may be strikingly reduced (12). However, a relationship between reduced skin blood flow and clinical sclerodermatous alterations has not been established (13).

A variety of pathogenetic, nonimmunological mechanisms have been suggested for the fibrosis of PSS, by analogy with other diseases in which abnormal fibrosis occurs. Among these diseases are phenylketonuria and the carcinoid syndrome. Studies in scleroderma patients have, however, revealed normal levels of urinary phenylalanine and its metabolites (14) and normal urinary excretion of serotonin degradation products (15). Similarly the vasospastic features of PSS, exemplified by the high incidence of Raynaud's syndrome, have suggested the possible pathogenetic influence of excessive circulating epinephrine or norepinephrine. Recent studies, however, have failed to show evidence of enhanced catecholamine production or excretion in PSS (16).

Autoantibodies are commonly observed in PSS. Antinuclear antibodies with either the speckled (17–20) or nucleolar pattern (21) are seen most frequently. Anti-RNA antibodies have been described (22), rheumatoid factors were found in 92 of 265 patients (23), and hypergammaglobulinemia was present in approximately one-half of patients (24). However, no correlation between the course or severity of PSS and the presence of autoantibodies has been observed (18, 19).

The not uncommon concurrence of polymyositis with PSS suggests the presence of disturbed cellular immunity in PSS, in view of the increasing evidence that polymyositis is associated with an abnormal cellular immune response to muscle (25–28). Infiltrates of mononuclear cells have been found in the skin and organs of

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Dr. Johnson's present address is the Division of Immunology, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267.

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[1] *Abbreviations used in this paper:* AS, active supernate, derived from PHA-P-stimulated peripheral blood mononuclear cells; CS, control supernate, derived from unstimulated peripheral blood mononuclear cells; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PHA, phytohemagglutinin; PSS, progressive systemic sclerosis; TCA, trichloroacetic acid; Tdr, thymidine.

PSS patients (2–10, 29). In rats with graft-versus-host disease, Stastny et al. (30) have observed scleroderma-like changes in the skin. A recent indication of the participation of abnormal cellular immunity in PSS has been furnished by Currie et al. (31), who observed that lymphocytes from PSS patients with myositis produced cytopathic alterations in fibroblast cultures.

In the present studies, normal human peripheral blood mononuclear cells have been stimulated with phytohemagglutinin (PHA)-P to produce lymphokine-rich (32) supernates, and the effect of these supernates on collagen synthesis has been examined in vitro. In appropriate dilution, enhanced collagen synthesis and accumulation in cultures of human fibroblasts have been observed, suggesting the presence of a fibroblast-stimulating factor in lymphokine-rich supernates.

## METHODS

*Preparation of active lymphokine-containing supernates.* The procedures utilized for the preparation of active supernatants (AS) by PHA-P (Difco Laboratories, Detroit, Mich.) and of control supernates (CS) by unstimulated mononuclear cells are presented in schematic form in Fig. 1. Human peripheral blood (donor 2) or leukocyte-rich buffy coats and plasma from packed red blood cell preparations (donors 1 and 3) from three unrelated normal donors were anticoagulated with heparin or acid citrate dextrose, respectively, diluted with an equal volume of normal saline, and centrifuged into a mixture (10:24, vol/vol) of 33.9% Hypaque (Winthrop Laboratories, New York, N. Y.) and 9% dialyzed, sterile Ficoll (Pharmacia, Uppsala, Sweden) according to the method of Böyum (33). The mixture of lymphocytes and monocytes thus obtained contained less than 1% polymorphonuclear leukocytes. The cells were washed three times in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.), containing in the first two washes 0.3 mM disodium-EDTA. They were then counted and divided between two groups of tubes. Each contained $5 \times 10^6$ mononuclear cells resuspended in 1 ml of RPMI-1640 containing 2 mM of glutamine, 90 μg of streptomycin, and 100 μg of aqueous penicillin. In some instances, human serum albumin (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.) was added to a final concentration of 0.2%. One group of tubes then received 40 μl of PHA dissolved in Hanks' balanced salt solution (HBSS), and the other group received HBSS alone. All tubes were then incubated at 37°C in an atmosphere of 95% air and 5% $CO_2$ for 1–1.5 h. To minimize the PHA content of the resulting AS, the cells were then washed three times and resuspended in medium 199 (Grand Island Biological Co.), containing 2 mM glutamine, 100 U/ml potassium penicillin G, and 90 μg/ml streptomycin. After incubation for 72 h, in the case of donors 1 and 3, or for 48, 72, and 120 h, in the case of donor 2, the supernates were removed by aspiration. At the conclusion of the incubation, the mononuclear cells were resuspended in 1 ml of RPMI-1640 with glutamine, 10% fetal calf serum (FCS), penicillin, and streptomycin, and pulse-labeled with 1 μCi/culture of tritiated thymidine (Tdr) ([*methyl*-$^3$H]thymidine, New England Nuclear, Boston, Mass. NET-027x) for 2 h. The nucleic acids were then extracted as previously described (34).

The mononuclear cell supernates were centrifuged, pooled, and passed through Millipore filters (Millipore Corp., Bedford, Mass.) of 0.22 μm pore size to free them from loose cells and cellular debris. Methylcellulose (Carbowax 6000, Applied Science Labs, Inc., State College, Pa.) was added at a concentration of 1% as a nontoxic surface active agent to prevent loss of proteins on glass and plastic surfaces (35). In the case of donor 2, 0.2% human serum albumin was present during the incubations, and in this circumstance Carbowax was not added. Dialysis was then performed overnight at 6°C against normal saline containing 1 mM sodium azide. All dialysis tubing was rendered nontoxic by methods previously described (26). AS from PHA-stimulated mononuclear cells and CS from unstimulated mononuclear cells were then independently passed through an immunoabsorbent consisting of an IgG fraction of rabbit antiserum against PHA, prepared by ethanol fractionation (36), covalently linked by the cyanogen bromide technique (37) to agarose (Bio-Gel A-5M, Bio-Rad Laboratories, Richmond, Calif.). The coupling phase was modified in this procedure, in that 0.1 M potassium phosphate buffer, pH 7.4, was used and the reaction was continued for 72 h. Elution of both AS and CS was performed with HBSS containing 1% Carbowax. The eluates were dialyzed against water, lyophilized, and reconstituted to the original volume of supernate with RPMI-1630, which contains no hydroxyproline. When no serum albumin was present, the medium was supplemented with 1% Carbowax. The preparations were then passed through Millipore filters (0.22 μM) for sterilization and stored at −70° until utilized. Supernates from PHA-stimulated mononuclear cells that had failed to show enhanced uptake of [$^3$H]Tdr were not used. Of five batches of mononuclear cells from four normal donors, incubated with PHA, there was no enhancement of [$^3$H]Tdr incorporation in two instances.

*Target cell system.* Human embryonic lung fibroblasts, WI-38 (38) (HEM Laboratories, Rockville, Md.), were obtained in confluent monolayers in Leighton tubes. The fibroblast monolayers received from the supplier varied in number of passages. No systematic investigation of responsiveness of the cultures to lymphocyte supernates based on passage number was performed, but on several occasions cultures from late passages spontaneously detached before the completion of the experiment. These experiments were not further evaluated. In vitro assays for the effects of mononuclear cell supernates upon these target cells were initiated in most cases on the 5th and 7th day after seeding of the cultures. Cultures were maintained until initiation of the assays in RPMI-1640 with supplement A, which reconstituted the medium to contain 20 mM Hepes buffer, 5 μg/ml ascorbic acid, 0.04 μg/ml methyl prednisolone, 10% inactivated FCS (Gibco), 1 μg $FeSO_4 \cdot 7\ H_2O$/ml, 2 mM glutamine, 100 U penicillin/ml, and 90 μg streptomycin/ml. Preliminary experiments demonstrated that FCS was essential for significant collagen synthesis. With 10% serum, collagen synthesis was enhanced by the addition of methyl prednisolone. Higher concentrations of methyl prednisolone were inhibitory. 2–4 h before assay, the fibroblast monolayers were washed three times. In experiments in which incorporation of [$^{14}$C]proline was not measured, the washed cells were then incubated with RPMI-1630 containing supplement A so as to provide an hydroxyproline-free medium. When the incorporation of [$^{14}$C]proline into protein was assayed, or when collagen content was measured by collagenase digestion, minimal Eagle's medium (Gibco) with supplement A was used, since this medium lacks both proline and hydroxyproline.

*Collagen assay.* Collagen accumulation by the human embryonic lung fibroblasts (WI-38) were measured as follows: The AS and CS preparations were dialyzed against minimal Eagle's medium (Gibco) containing supplement A (see above) without fetal calf serum. After dialysis, inactivated fetal calf serum was added to a concentration of 10% and the preparations were variably diluted with fresh minimal Eagle's medium containing supplement A. Three types of experiments were utilized to determine collagen content or synthesis. In the first, presented in Tables IV and V, the effect of supernates upon the intracellular and adherent collagen content of the monolayers was assayed. In these experiments, Leighton tube monolayers (1 × 4 cm) were incubated with the appropriate dilutions of AS and CS either in static concentrations for 48 h (Table IV) or in progressive daily dilutions from an initial 1:8 dilution during the first 24 h to a final concentration of 1:1,944 on the 6th day (Table V). At the completion of the culture period, supernates were removed by aspiration and the monolayers gently washed by two additions of 1 ml of 37°C HBSS. The monolayers were then hydrolyzed by the addition of 3 ml of 6 M HCl for 15 h at 130°C in a heating block, while sealed in the original Leighton tube with screw caps with Teflon seals. The collagen content was then assayed by spectrophotometric determination of hydroxyproline in the neutralized hydrolysates by the method of Prockop and Udenfriend (39), as modified for smaller samples by LeRoy et al. (40).

The second type of assay (Tables VIII–X), which utilized the incorporation of [$^{14}$C]proline into collagen as a direct measure of collagen synthesis, was carried out as follows: 2.5 $\mu$Ci of [$^{14}$C]proline (L-[U-$^{14}$C]proline, New England Nuclear, NEC-285) were added to each fibroblast monolayer containing either AS or CS for 4 h. At the termination of the experimental period, the supernates were removed quantitatively by four washes of 0.5 ml each of warm saline. One drop of FCS was then added to each supernate to facilitate precipitation of proteins upon subsequent addition of an equal volume of 10% trichloroacetic acid (TCA)-containing 0.5% tannic acid. The supernate was then removed by centrifugation and the precipitate resuspended by vortexing in 3 ml of 5% TCA-0.25% tannic acid, centrifuged, and again resuspended by vortexing in 5% TCA-0.25% tannic acid. A final washing of the resulting precipitate was similarly performed with 5% TCA alone. The washed precipitates were then dissolved in 0.7 ml of 0.5 N NaOH by being heated to 56°C and vortexed. The solutions were then transferred quantitatively to Pyrex screw-top centrifuge tubes with two washes each of 0.4 ml of concentrated HCl and a third 0.5-ml wash with 6 N HCl. The tubes were then sealed tightly and hydrolyzed as described above. The washed fibroblast monolayers remaining, after removal of the supernates described above, were loosened from the glass surfaces by the addition of 0.5 ml of 0.5 N NaOH after each tube had received 1 drop of FCS. When the solutions had clarified, they were transferred quantitatively, by two washes of 1.25 ml of water for each Leighton tube. The resultant solutions, containing dissolved monolayer cells and previously insoluble adherent

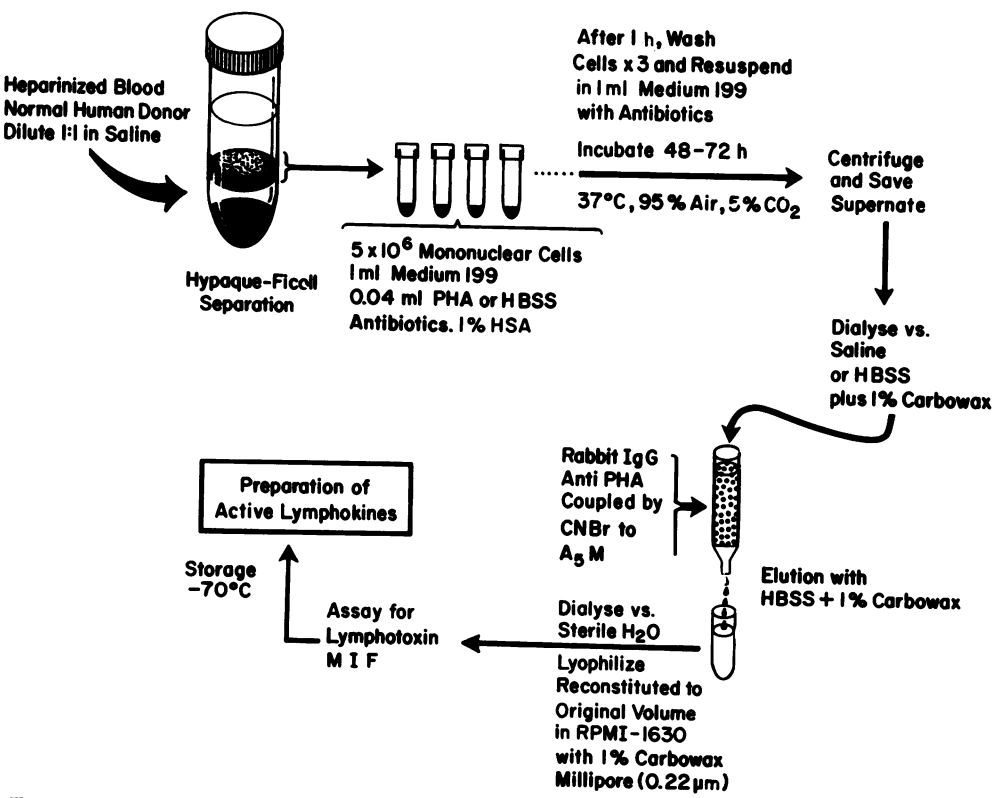


FIGURE 1 Schematic representation of the method of preparation of active lymphokine-containing supernates. The preparation of CS and AS differs only in that the former received no PHA-P. HSA, human serum albumen; MIF, migration-inhibitory factor.

collagen, were processed as described above for the supernates by TCA-tannic acid precipitation of proteins and subsequent hydrolysis. In some instances (Tables I, VIII), only soluble supernate collagen was determined. Assay for [$^{14}$C]hydroxyproline was performed as described by Juva and Prockop (41). 2 mg of hydroxyproline and 1 mg of proline were added to each sample before hydrolysis for subsequent calculation of recovery and as carriers for the [$^{14}$C]proline and [$^{14}$C]hydroxyproline contained in the hydrolysates.

A third type of collagen assay was performed with a highly specific clostridial collagenase (42, 43). In this technique, chromatographically purified collagenase (Worthington Biochemical Corp., Freehold, N. J.) was subjected to G-200 (Pharmacia) gel chromatography and the fractions were assayed for collagenase activity and noncollagenase protease activity (43). Those fractions showing collagenase activity against undenatured bovine tendon collagen, but only limited noncollagenase protease activity against bovine casein, were pooled and then alkylated with *N*-alpha-tosyl-L-lysine (Calbiochem, San Diego, Calif.) and dialyzed (43). This preparation was used in the assay described by Peterkofsky and Diegelmann (42) to measure the soluble [$^{14}$C]-proline and [$^{14}$C]hydroxyproline released by collagenase digestion of the precipitate obtained with TCA from supernates and suspensions of monolayers of fibroblast cultures containing either AS or CS (Table XI). Monolayers were loosened from the Leighton tubes by treatment with 0.25% trypsin (Grand Island Biological Co.) and washed in HBSS, and the fibroblasts in a 0.1 ml portion were counted in a hemocytometer. To the remaining cells was then added an equal volume of 0.5 N NaOH. The mixture was then processed by threefold TCA-tannic acid precipitation and subsequent collagenase digestion. This type of assay reflects collagen content by specific release of TCA-soluble peptides from collagen.

In some experiments, an attempt was made to evaluate the possibility that the action of AS was to impair degradation of collagen rather than to enhance synthesis. WI-38 fibroblast cultures were prelabeled by incubation for 19 h in Eagle's medium containing supplement A and 4 μCi/Leighton tube of [$^{14}$C]proline to prelabel the supernate and monolayer collagen (Table XII). AS and CS were then added to the prelabeled cultures in RPMI-1640 for 4 h to result in a final concentration of 1:12 in 1.5 ml of medium containing sufficient hydroxyproline (1 mg/ml) and proline (2 mg/ml) to diminish markedly the further incorporation of [$^{14}$C]proline. Monolayers and supernates, combined, were submitted to TCA precipitation and determination of collagen content by the Juva and Prockop assay (41).

*Proliferation of fibroblasts.* Proliferation of fibroblasts was determined from the incorporation of [$^{3}$H]Tdr into DNA over 4 h with 2–4 μCi/Leighton tube. Extractions and scintillation counting were carried out as described by Douglas et al. (34).

*Noncollagenous protein synthesis.* Noncollagenous protein synthesis was determined by measuring the incorporation of a reconstituted $^{14}$C-amino acid hydrolysate (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) into TCA-precipitable protein remaining insoluble after boiling for 30 min (44).

*Evaluation of completeness of removal of PHA from supernates.* AS and CS supernates were tested for the completeness of removal of PHA with $^{125}$I-labeled (carrier-free $^{125}$I, New England Nuclear) PHA labeled by the method of Taurog (45) with lactoperoxidase (Calbiochem). To 20-ml volumes of 72-h AS and CS supernates, 25 μl of

TABLE I

*Effect of PHA on Collagen Accumulation by Human Fibroblasts*

| Culture medium | Collagen content ([$^{14}$C]hydroxyproline) | Decrease |
|---|---|---|
| | *cpm* | % |
| Eagle's | 11,431 | |
| | 11,804 | |
| | 11,042 | |
| Mean | 11,429 | |
| | | 64.7 |
| Eagle's + PHA | 3,557 | |
| | 3,857 | |
| | 5,082 | |
| | | $P < 0.001$ |
| Mean | 4,165 | |

Effect of PHA-P (40 μl/ml) upon collagen accumulation by human WI-38 fibroblasts. Fibroblast supernates were precipitated with 5% TCA and 0.5% tannic acid and hydrolyzed in 6 N HCl, and radioactivity of [$^{14}$C]proline incorporated into [$^{14}$C]hydroxyproline was measured.

$^{125}$I-PHA/ml were then added, containing a total of 305,875. cpm. The supernates were each mixed with 40 ml of a rabbit anti-PHA immunoabsorbent in which the PHA was covalently linked by cyanogen bromide to Sepharose 4B, as described by Cuatrecasas et al. (37), and allowed to incubate for 2 h at 6°C. Each supernate was then added to the top of a presettled column containing 20 ml of the immunoabsorbent. After collection of the void volume, the effluents, including one column volume of eluant (HBSS containing 1% Carbowax) were recovered and pooled. The pooled effluents were then dialyzed against sterile water, lyophilized, and reconstituted to the original 20-ml volume in RPMI-1630. A portion of each was counted in a gamma counter (Tracerlab Div., LFE Electronics, Richmond, Calif.). The columns were then washed with three column volumes of saline and eluted with three column volumes (180 ml total) of pH 3.0, 0.1 M glycine-HCl buffer. The pooled effluent was then dialyzed against water, lyophilized, and reconstituted to 20 ml in saline, and a portion was counted in a gamma counter.

## RESULTS

*Preparation of active and control supernates.* Lymphokine-rich AS's and CS's were generated by exposure of peripheral blood mononuclear cells to PHA or HBSS (Fig. 1). In these experiments the PHA-containing RPMI-1640 medium was left in contact with the cells for 90 min, after which the cells were washed and the medium was replaced. The supernates, after incubation, were then further processed by immunoabsorption to remove trace amounts of PHA. The necessity of this step is demonstrated in Table I, in which the effects of PHA upon collagen synthesis are examined. In this experiment, the incorporation of [$^{14}$C]proline into col-

TABLE II

*Removal of PHA from Mononuclear Leukocyte Culture Supernates by Immunoabsorption**

| Sample | $^{125}$I-PHA-P |
|---|---|
| | *cpm* |
| $^{125}$I-PHA-P added to AS and CS | 306 × 10$^6$ |
| CS‡ after immunoabsorption | 60 (49–78) |
| AS§ after immunoabsorption | 200 (85–346) |
| Glycine-HCl eluate from AS columns | 200 × 10$^6$ (155–265 × 10$^6$) |

* Recovery of $^{125}$I-trace-labeled PHA-P radioactivity after elution from an immunoabsorbent consisting of rabbit anti-PHA-P covalently linked to A-5M agarose by cyanogen bromide conjugation. Results represent the means of three experiments.

‡ CS derived from peripheral blood mononuclear cells in HBSS.

§ AS derived from peripheral blood mononuclear cells treated with PHA.

lagen-derived [$^{14}$C]hydroxyproline by human embryonic WI-38 lung fibroblasts was measured in the presence of 4 μl PHA/ml of medium. This was an amount equal to one-tenth of the concentration of PHA present in the CS's during the initial incubation with peripheral blood leukocytes for the preparation of lymphokine solutions. The effects of PHA at this concentration are shown in Table I. It is clear that low concentrations of PHA reduced collagen accumulation, thus indicating the desirability of removing PHA from the test supernates. It should be pointed out, however, that in the preparation of AS the peripheral blood leukocytes were washed after initial exposure to PHA and resuspended without additional PHA.

*Adequacy of removal of PHA from supernates.* An immunoabsorbent consisting of an IgG fraction of rabbit anti-PHA linked to Sepharose 4B was used to remove PHA. To test the adequacy of the immunoabsorbent, $^{125}$I-PHA was added to both AS and CS. In a representative experiment, 20 ml of pooled AS and CS were individually passed through the immunoabsorbent and the eluates were dialyzed, lyophilized, and reconstituted to their original volume. An aliquot of each was counted and compared with the total radioactivity of $^{125}$I-PHA applied to the column and present in the fraction subsequently eluted from the immunoabsorbent with glycine-HCl buffer. In Table II it can be seen that the PHA was quantitatively removed by this procedure.

*Evaluation of lymphokine activity in mononuclear cell supernates.* Proliferation inhibitory factor was demonstrated in AS. This was present to a dilution of 1:16 in a preparation from donor 1 as measured by Badger, et al. (46). Undiluted CS preparations were slightly inhibitory in comparison with control medium (no mononuclear cells present during a 48-h incubation), but none showed inhibition at a dilution of 1:2 or greater. The presence of cloning inhibitory factor, assayed by comparison of the ability of AS and CS to suppress the establishment of clones of WI-38 fibroblasts seeded into tissue culture flasks, as described by Lebowitz and Lawrence (47), was also detectable in the same AS preparation at a dilution of 1:12. Dilutions of CS of 1:2 and more similarly did not suppress the establishment of fibroblast clones. Migration inhibitory factor was detectable in undiluted AS but not in CS, by the method of David and David (48).

CS and AS were also tested for lymphotoxin activity against mouse L-929 fibroblasts (44). A pool was made of AS from both at 48-h and 72-h culture of mononuclear cells from the same source, and processed as described above, with removal of PHA. The lymphotoxin activity of these supernates was compared by the method of Granger and Kolb (44). The AS pool, as seen in Table III, caused a significant decrease in the synthesis of cellular protein by L-929 mouse fibroblasts.

*Stimulation of collagen accumulation by human fibroblasts measured as hydroxyproline content.* Various concentrations of AS and CS (donor 1) in fresh hydroxyproline-free RPMI-1630 were applied to WI-38 fibroblasts grown to confluence in Leighton tubes. The effects upon proliferation, noncollagenous protein synthesis, and collagen content of the monolayers were determined.

In initial experiments, AS or CS, undiluted and diluted 1:4, were added to each tube of fibroblasts. At the end of 48 h of incubation, 0.75 μCi of reconstituted $^{14}$C-amino acid hydrolysate was added to one set each of the AS- and CS-containing cultures and incubation was allowed to proceed for 1 h in amino acid-free medium. To another set of Leighton tubes, 2 μCi of [$^3$H]Tdr was added and labeling was allowed to proceed for two h to

TABLE III

*Lymphotoxin Activity of Purified Supernates after Stimulation of Human Mononuclear Cells by PHA**

| Supernate after immunoabsorbent processing | $^{14}$C-amino-acid incorporation | Decrease in $^{14}$C-amino acid‡ incorporation |
|---|---|---|
| | *cpm* | % |
| CS-pool§ | 4,833 | 27 |
| AS-pool§ | 3,517 | |

* Demonstration of the lymphotoxin activity against L-929 mouse fibroblasts by supernate from PHA-P-stimulated mononuclear cells (AS) after immunoabsorption, dialysis, and reconstitution. Results represent mean of three determinations.

‡ % decrease = [(cpm CS − cpm AS)/cpm CS] × 100.

§ Equal volumes of CS harvested at 48 and 72 h were mixed and compared with a mixture of equal volumes of AS harvested at 48 and 72 h.

assay proliferation. A third set of fibroblasts was washed in warm HBSS, hydrolyzed in 6 N HCl, and assayed for hydroxyproline content to determine cellular and insoluble collagen. It can be seen that with undiluted AS and CS from donor 1 (Table IV), cellular proliferation and noncollagenous protein accumulation were markedly reduced while the collagen content, expressed as hydroxyproline content of the AS-treated monolayers, was larger than that of the CS-treated monolayers. The enhancement of collagen content was significantly increased ($P < 0.005$) when the supernate was diluted 1:4. As a readily apparent loss of cells from the monolayers treated with AS was seen through the inverted microscope, it appeared that decreased numbers of surviving cells were accumulating collagen in increased amounts. The decrease in fibroblasts was indicated by gaps in the cellular mat. Such decreases were regularly seen in fibroblast cultures in the presence of dilutions of AS of 1:16 or less. These cells could not actually be counted because many of the fibroblasts remaining in the AS-treated tubes, particularly in lower dilutions, were very fragile when resuspended.

In another experiment, more dilute preparations of AS and CS (donor 1) were used in a schedule designed to resemble the sequence of events in an inflammatory reaction. In this experiment an initial, relatively high concentration of AS and CS was diluted daily over 6 days, in an attempt to parallel a falling concentration of mediators as might be expected in tissue with the passage of time. In this experiment, the dilution of AS and CS in the first 24 h was 1:8. The dilution was progressively increased threefold at daily intervals, to a final dilution of 1:1,944 on the 6th day of incubation without fresh addition of AS or CS. On the 7th day, cellular proliferation, noncollagenous protein synthesis, and hydroxyproline content were measured, as in the two previous experiments. The results are given in Table V. Whereas the inhibitory effect of the active lymphokine-rich AS upon cellular proliferation remained, there was no significant difference in noncollagenous protein synthesis. However, there was a highly significant increase in the collagen content of the monolayers, as indicated by the fact that the AS-treated fibroblasts had accumulated 80% more collagen than the control fibroblasts.

In a further experiment designed to estimate the loss of fibroblasts from the monolayers, various dilutions of AS (donor 1) were incubated with fibroblast monolayers in dilutions rainging from 1:1 to 1:32 for 2 days. In addition, progressive threefold daily dilutions of AS and CS were made from a dilution of 1:8 to a final dilution of 1:1,944 over 6 days. Comparisons of the loss

TABLE IV

*Effect of AS and CS upon Cellular Proliferation, Noncollagenous Protein Synthesis, and Collagen Content of Fibroblast Monolayers**

| Supernate | Cell proliferation | Noncollagenous protein | Collagen |
|---|---|---|---|
| | *cpm [$^3H$]Tdr* | *$^{14}C$ cpm* | *μg hydroxyproline* |
| CS (undiluted) | 3,391 | 18,928 | 1.12 |
| | 8,665 | 23,898 | 1.39 |
| Mean | 6,028 | 21,412 | 1.26 |
| AS (undiluted) | 1,325 | 2,801 | 1.21 |
| | 1,344 | 2,852 | 1.76 |
| Mean | 1,335 | 2,827 | 1.49 |
| Difference‡, % | −78 | −87 | +18 |
| CS (1:4 dilution) | 1,564 | 6,797 | 0.9 |
| | 1,602 | 6,932 | 0.5 |
| | 1,591 | 11,398 | § |
| Mean | 1,586 | 8,376 | 0.7 |
| AS (1:4 dilution) | 619 | 7,794 | 1.2 |
| | 1,381 | 8,069 | 1.3 |
| | | 10,774 | 1.3 |
| Mean | 1,000 | 8,879 | 1.3 |
| Difference‡, % | −37 | +6 | +86 ($P < 0.005$) |

* Corrected for hydroxyproline content at initiation of experiment (0.99 μg).
‡ [(AS) − (CS]/(CS) × 100.
§ Sample lost during hydrolysis.

TABLE V

*Effects of Progressive Daily Dilutions of AS and CS upon Cellular Proliferation, Noncollagenous Protein Synthesis, and Collagen Content of Fibroblast Monolayers*

| Supernate‡ 1/8 1/1,944 | Cell proliferation | Noncollagenous protein | Collagen* |
|---|---|---|---|
| | *[$^3H$]Tdr cpm* | *$^{14}C$ cpm* | *μg hydroxyproline* |
| CS | 13,148 | 13,400 | 1.39 |
| | 12,324 | 7,013 | 1.18 |
| | 13,494 | 13,867 | 1.54 |
| | 11,917 | 18,299 | 1.40 |
| | 11,470 | 13,722 | 1.44 |
| Mean | 12,471 | 13,260 | 1.39 |
| AS | 9,573 | 16,414 | 2.36 |
| | 8,081 | 17,852 | 2.27 |
| | 9,228 | 13,909 | 2.05 |
| | 9,022 | 17,604 | 2.01 |
| | 9,576 | 17,156 | 2.49 |
| Mean | 9,096 | 16,587 | 2.49 |
| Difference§, % | −27 | +25 | +80 |
| *P* | <0.001 | >0.10 | <0.001 |

* Corrected for content of hydroxyproline at initiation of experiment (0.99 μg). Assays were performed on 7th day.
‡ Supernates were progressively diluted threefold daily over 5 days from an initial dilution of 1:8.
§ Calculated as in Table IV.

TABLE VI

*Effects of AS and CS at Selected Dilutions upon Loss of Fibroblasts from the Monolayers*

| Supernate* | Dilution of supernates | Incubation period | Loss of fibroblasts from monolayer‡ |
|---|---|---|---|
| | | *days* | |
| AS | 1:1 | 2 | + + |
| AS | 1:4 | 2 | + + |
| AS | 1:8 | 2 | + |
| AS | 1:16 | 2 | + |
| AS | 1:32 | 2 | None |
| AS | 1:8 → 1:1,944§ | 6 | None |
| CS | Undiluted | 2 | None |
| CS | 1:8 → 1:1,944§ | 6 | None |

* Triplicate samples examined.

‡ Visual approximation by light microscopic examination of monolayers at conclusion of experiment. + + indicates approximately 50% of cells remaining intact. + indicates approximately 75% of cells appear intact. If the monolayer remained confluent, it was graded as having no loss. Fibroblasts remaining attached to the Leighton tube were enumerated at a magnification of 200×.

§ Progressively diluted threefold every day over a 6-day period.

of fibroblasts from the monolayers were made in the AS and CS cultures. In this experiment, triplicate fibroblast preparations were stained with hematoxylin and eosin. Total numbers of cells in high-power light microscopic fields were counted. Loss of up to 25% of fibroblasts from the monolayers, as compared with the control monolayers, was arbitrarily described as 1+ cellular loss and a 26–50% loss of fibroblasts was recorded as a 2+ loss. The results are shown in Table VI: as the dilution of AS increased, loss of fibroblasts from the monolayer was less apparent. At a dilution of AS of 1:32, there was no difference in numbers of attached cells in the AS and CS cultures at 48 h. In the above experiments, therefore, treatment of fibroblasts with active lymphokine-rich supernates appears to result in a selected, surviving population of fibroblasts that accumulate more collagen per cell than the fibroblasts in the control cultures.

*Stimulation of collagen synthesis measured by [$^{14}C$]-proline incorporation.* In the next series of experiments, uniformly labeled [$^{14}$C]proline was utilized to measure the rate of accumulation of [$^{14}$C]hydroxyproline in collagen of the supernates of fibroblast cultures. In these experiments protein was separated from smaller molecular weight polypeptides and amino acids in the supernates by TCA-tannic acid precipitation. In the first of these studies, the mononuclear cells from a Hypaque-Ficoll preparation of normal human peripheral blood leukocytes (donor 2) were freed of adherent cells by suspending the mononuclear cells in RPMI-1640 with 5% inactivated FCS and permitting them to adhere to glass in Leighton tubes for 1 h at 37°C. In this experiment, $10 \times 10^6$ mononuclear cells were added to each of a series of Leighton tubes with an available surface area of 4 cm$^2$. 51% of the cells, or approximately $5 \times 10^6$/Leighton tube, became adherent. The nonadherent cells from these tubes were then pooled and further exposed to plastic in Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) for an additional hour. The remaining nonadherent cells were morphologically all small lymphocytes and are referred to in Table VII as nonadherent mononuclear cells. These were then divided into lots of $5 \times 10^6$ lymphocytes/tube in 1.0 ml of medium 199. In another series of tubes, $5 \times 10^6$ mononuclear cells, separated by the Hypaque-Ficoll method only, were suspended in 1 ml of medium 199. These are referred to in Table VII as whole mononuclear cells. These two cell populations were then each divided into two groups, one receiving PHA made up in HBSS and the other only HBSS. Cultures were incubated for 1.5 h and then, after the washing out of unbound PHA, they were incubated for 120 h more in medium 199. At intervals of 48, 72, and 120 h the supernates were removed and fresh medium containing 0.2% human serum albumin was substituted. After 120 h the cells were labeled with [$^3$H]Tdr for 1 h after being resuspected in 1 ml each of RPMI-1640 containing 10% inactivated PCS. The ratios of [$^3$H]Tdr incorporation of the stimulated to unstimulated cultures are given in Table VII. The relatively low stimulation ratios observed reflect previous findings (49) that continuous presence of PHA is necessary for maximum stimulation; and as is well established, maximal stimulation indices occur at 48–72 h with PHA (50). The supernates from these cultures were then treated by batch

TABLE VII

*Comparison of the DNA Stimulation Indices of Unseparated and Nonadherent Mononuclear Cells Exposed to PHA or HBSS and Radiolabeled by Tritiated Thymidine Incorporation*

| Mononuclear cell type | Number of determinations | Mean stimulation index* |
|---|---|---|
| Whole mononuclear cells | 4 | 1.6 |
| Nonadherent | 8 | 4.3 |

Tritiated thymidine incorporation into DNA was assayed on 5th day. 40 μl of HBSS was added to control cells and 40 μl of PHA-P added to stimulated cells.

* Index calculated from [(cpm of stimulated cells − cpm of unstimulated cells)/cpm of unstimulated cells] × 100.

absorption with anti-PHA immunoabsorbent and dialyzed against RPMI-1630 containing antibiotics. After dialysis, a portion of each pool of AS and CS was diluted 1:12 in fresh Eagle's medium containing supplement A and added to triplicate WI-38 fibroblast cultures for 24 h. The medium was then replaced with fresh medium of the same composition, containing [$^{14}$C]proline. After an additional 24 h, the CS were extracted by the Juva and Prockop technique (41) for the measurement of collagen accumulation as described above. As shown in Table VIII, supernates from lymphocytes freed of adherent cells enhanced collagen accumulation, although the increment was small. Supernates from the stimulated whole mononuclear cell populations that contained both adherent and nonadherent mononuclear cells produced a greater increment in collagen accumulation. This suggests that, as in the production of the lymphokine, lymphotoxin (51), the cooperative interaction of adherent cells, and T-lymphocytes may promote synthesis or release of the factor enhancing collagen accumulation.

TABLE VIII

*Effects of Various Supernates from Human Peripheral Blood Mononuclear Cells upon Collagen Synthesis by Human (WI-38) Fibroblasts as Measured by Incorporation of [$^{14}$C]Proline into Soluble Collagen**

| Source of mononuclear cell supernate | Soluble collagen content‡ | Increase in collagen accumulation§ |
|---|---|---|
| | [$^{14}$C]*hydroxyproline cpm* | % |
| Nonadherent cells | | |
| + HBSS‖ | 11,112 | |
| | 10,983 | |
| | 10,456 | |
| Mean | 10,850 | 13 |
| Nonadherent cells | | |
| + PHA | 12,630 | |
| | 11,995 | $P < 0.05$ |
| | —¶ | |
| Mean | 12,313 | |
| Whole mononuclear cells | | |
| + HBSS | 9,356 | |
| | 8,824 | |
| | —¶ | |
| Mean | 9,090 | 47 |
| Whole mononuclear cells | | |
| + PHA | 14,022 | |
| | 11,619 | |
| | 14,576 | $P < 0.05$ |
| Mean | 13,405 | |

* Mononuclear cell supernates were diluted 1:12 in collagen labeling medium and exposed to WI-38 fibroblasts for 48 h before termination of assay.

‡ Soluble, supernate collagen assayed on 5% TCA and 0.5% tannic acid precipitates by the Juva-Prockop method (41).

§ Percent increase in accumulation of collagen: {[cpm[$^{14}$C]-OH-proline(AS) − cpm [$^{14}$C]OH-proline (CS)]/cpm[$^{14}$C]-OH-proline(CS)} × 100.

‖ All supernates diluted 1:12.

¶ Sample lost during hydrolysis.

TABLE IX

*Soluble Supernate and Monolayer Collagen Accumulation by Human Fibroblasts (WI-38) during Exposure to CS and AS**

| Supernate added (1:12) | Collagen sample | Collagen content‡ | Increment§ |
|---|---|---|---|
| | | [$^{14}$C]*hydroxyproline cpm* | % |
| CS | Supernate | 9,600 | |
| | | 9,600 | |
| | | 11,840 | |
| | | 7,600 | +31 |
| Mean | | 9,660 | |
| AS | Supernate | 12,560 | |
| | | 11,840 | $P < 0.058$ |
| | | 11,040 | |
| | | 15,280 | |
| Mean | | 12,680 | |
| CS | Monolayer | 1,961 | |
| | | 3,101 | |
| | | 1,343 | |
| | | 1,631 | +157 |
| Mean | | 2,009 | |
| AS | Monolayer | 5,176 | |
| | | 5,472 | $P < 0.01$ |
| | | 3,368 | |
| | | 6,633 | |
| Mean | | 5,162 | |

* Assay period 0–4 h.

‡ Preparation of supernate carried out as in Table VII.

§ Calculated as in Table VIII.

In the next group of experiments, the effects of AS and CS prepared from whole mononuclear cell populations upon soluble and insoluble collagen accumulation in the fibroblast cultures were assayed during a 4-h labeling interval, indicated in Table IX. AS and CS (donor 1), diluted 1:12, were added to fibroblast monolayers. Four tubes in each of the experimental and control groups were pulse-labeled with [$^{14}$C]proline during this labeling interval. Increases in collagen accumulation were observed in both the supernate ($P < 0.058$) and the insoluble and cellular fractions ($P < 0.01$).

*Effects of AS and CS upon collagen degradation.* Inasmuch as the $^{14}$C-amino acid-labeled collagen measured in these studies represented the difference between synthesis and degradation (52), an experiment was performed in which WI-38 fibroblasts were prelabeled for

TABLE X

*Comparison of the Effects of CS and AS* on the Degradation of Prelabeled Collagen‡ in the Combined Monolayer and Supernate Fractions*

| Supernate added (1:12) | Collagen content§ | Difference‖ |
|---|---|---|
| | [$^{14}$C]*hydroxyproline cpm* | % |
| CS | 48,216 | |
| | 45,039 | |
| | 62,679 | |
| | 35,923 | |
| | 36,065 | |
| | 48,157 | |
| | 41,761 | |
| Mean | 45,406 | +5 (NS) |
| AS | 39,374 | |
| | 66,895 | |
| | 45,495 | |
| | 38,410 | |
| | 47,052 | |
| | 49,953 | |
| | 46,082 | |
| Mean | 47,609 | |

* AS was derived from PHA-P (40 μl)-treated mononuclear cells dissolved in HBSS and CS derived from HBSS (40 μl)-treated mononuclear leukocytes.

‡ Human WI-38 fibroblast collagen was prelabeled by incubation of monolayers with [$^{14}$C]proline overnight.

§ Preparation of combined supernate and monolayer fractions was performed as in Table IX after loosening of cell mat with 0.5 N NaOH.

‖ Calculated as in Table VIII.

19 h with 4 μCi of [$^{14}$C]proline/tube. Excess cold proline and hydroxyproline were then added to stop further incorporation of label, and AS and CS (donor 1) in RPMI-1640 were then added to the labeling medium to a final dilution of 1 : 12. The mixtures were then incubated for 4 h. The results of this study are shown in Table X. The values reported, which represent the TCA-tannic acid-precipitable counts remaining at the end of this 4-h period, were determined on the entire contents of each of six Leighton tubes containing either AS or CS (both 1 : 12), thereby combining the supernate and monolayer contributions. No significant difference between AS and CS-treated cultures was noted, confirming by this assay that AS did not act by impairing degradation of collagen.

Since the effect of the active factor in the AS might be reflected in the degree of hydroxylation of proline within the synthesized collagen, a final group of experiments was carried out in which the total [$^{14}$C]proline and [$^{14}$C]hydroxyproline incorporated into supernate and monolayer collagen was measured. In these experiments correction was also made for the fibroblasts surviving at the end of the 4-h labeling period. Highly purified collagenase was added to portions of total TCA-tannic acid-precipitable protein from radiolabeled fibroblast supernates and monolayers and assayed by the method of Peterkofsky and Diegelmann (42). The radioactivity rendered soluble to the TCA-tannic acid precipitant after collagenase digestion in these experiments represents digested collagen, and contains both [$^{14}$C]proline and [$^{14}$C]hydroxyproline, since the latter is not specifically isolated as in the prior experiments. In these experiments, the fibroblasts were incubated with 1 : 16 dilutions of AS and CS (donor 3) for 44 h. At the end of this time, [$^{14}$C]proline labeling was performed for 4 h. The supernates were then harvested and the monolayers rinsed several times with HBSS at 37°C. The fibroblasts were then suspended by treatment with a 0.25% trypsin solution and divided into portions for determinations of cell counts and collagen content. For the determination of collagen content, i.e. collagenase-sensitive protein, after removal of a 20% portion of suspended fibroblasts, the remaining fibroblasts were combined with washes from the parent Leighton tubes with 0.5 N NaOH in a total volume equal to that of the fibroblast suspension. The mixture was then processed by TCA-tannic acid precipitation and subsequent collagenase digestion. The CS were similarly processed without the addition of NaOH. The results for the fibroblast monolayers and supernates are shown in Table XI.

Table XI shows that there were fewer viable AS-treated fibroblasts at the end of the assay period. There was no difference in the total $^{14}$C-radioactivity content of the portions subjected to collagenase digestion. There was a small, but not significant, decrease in the monolayer collagen, as measured by collagenase solubilization of $^{14}$C-radioactivity of proline and hydroxyproline. However, when these values were corrected to reflect the collagen accumulation per cell at the time of assay, i.e., during the 45th through 48th h, there was a significant enhancement of collagen accumulation in the AS-treated fibroblast monolayers. Similar results are demonstrated more strikingly in the case of the soluble or supernate collagen of this experiment, both without and with correction for viable fibroblasts at the end of the experiment. It is noteworthy here that significantly greater accumulation of collagen was found in the AS-treated fibroblasts when expressed as the total amount of collagen solubilized by collagenase, in spite of the lower numbers of cells surviving in these cultures.

## DISCUSSION

There is considerable evidence for abnormal collagen accumulation in PSS (2–10). In this disease, normally

TABLE XI

*A Comparison of the Cellular and Soluble Collagen Accumulation by Human Fibroblasts (WI-38) when Treated with AS or CS and Assayed by Collagenase Digestion*

| Mononuclear cell supernate (1:16) | Collagen sample | Fibroblast count | Total radioactivity before collagenase‡ | Collagenase solubilized | Collagenase solubilized | Increment§ |
|---|---|---|---|---|---|---|
| | | $\times 10^{-5}$ | $^{14}C$ cpm | $^{14}C$ cpm | cpm/$10^5$ fibroblasts | % |
| CS | Monolayer | 4.2 | 41,632 | 7,768 | 1,849 | |
| | | 4.4 | 49,568 | 7,472 | 1,698 | |
| | | 4.8 | 46,656 | 8,580 | 1,788 | |
| | | 5.5 | 48,160 | 7,432 | 1,351 | |
| Mean | | 4.7 | 46,504 | 7,813 | 1,672 | |
| | | | | | | +27 |
| AS | Monolayer | 3.8 | 49,088 | 7,176 | 1,888 | |
| | | 3.5 | 38,080 | 7,692 | 2,198 | |
| | | 3.6 | 48,928 | 8,080 | 2,244 | |
| | | 3.0 | 45,280 | 6,568 | 2,189 | |
| Mean | | 3.5 | 45,344 | 7,379 | 2,130 | |
| *P* | | 0.01 | 0.72 | 0.34 | 0.016 | |
| CS | Supernate | 4.2 | 143,360 | 24,256 | 5,575 | |
| | | 4.4 | 149,408 | 25,516 | 5,799 | |
| | | 4.8 | 174,176 | 25,496 | 5,312 | |
| | | 5.5 | 162,528 | 28,160 | 5,120 | |
| Mean | | 4.7 | 157,368 | 25,857 | 5,452 | |
| | | | | | | +68 |
| AS | Supernate | 3.8 | 157,568 | 30,998 | 8,155 | |
| | | 3.5 | 147,584 | 33,012 | 7,860 | |
| | | 3.6 | 175,872 | 33,636 | 9,343 | |
| | | 3.0 | 143,648 | 29,776 | 9,925 | |
| Mean | | 3.5 | 156,168 | 31,853 | 9,258 | |
| *P* | | 0.01 | 0.91 | 0.003 | 0.001 | |

* Fibroblasts counted at termination of experiment are recorded as viable, i.e. trypan blue dye-excluding, cells at the end of 48 h.

‡ [$^{14}C$]Proline labeling was performed during the final 4 h of exposure to AS and CS (45th through 48th h), on TCA-tannic acid precipitable protein.

§ Calculated as in Table VIII.

loose supporting connective tissue is replaced by dense collagen. There are cogent reasons to invoke an immune mechanism in this disease. Chronic mononuclear infiltrates are frequently seen in association with fibrosis in diverse conditions such as chronic hepatitis, tuberculosis, sarcoidosis, and polymyositis. They are also commonly observed in the circumscribed scleroderma-like disease, morphea (53). In each of these, cell-mediated immune mechanisms have been implicated in the evolution of the disease process. In addition, perivascular lymphocytic infiltrates have been observed in the dermis of patients with active scleroderma when biopsies were taken from edematous or erythematous areas of skin or at the margin between sclerodermatous and normal skin in patients with active scleroderma.[2]

[2] Gilliam, J., and M. Ziff. Unpublished observations.

Mediators of cellular immunity, such as lymphotoxin (44), proliferation inhibitory factor (46), and cloning inhibitory factor (47), encompass activities capable of impairing the viability and capacity for repair of a variety of target cells. They are known to coexist in supernates of mitogen-stimulated lymphocytes, although isolation techniques have not yet established whether they are discrete proteins (54). However, no immunological mediator capable of eliciting the enhanced accumulation of collagen by fibroblasts has been previously described, although a number of indirect observations have suggested the existence of factors derived from leukocytes that promote fibroblast growth or function. Carrel reported in 1922 (55) that chicken leukocytes derived from either inflamed connective tissue or from peritoneal exudates caused an enhancement of the growth

of chick fibroblasts in tissue culture. Castor and coworkers (56–59) have described a "connective tissue-activating peptide" found in normal and variously stimulated leukocytes as well as in inflammatory lesions such as the cotton pellet granuloma. This factor increases the metabolic activity of human fibroblasts in tissue culture, including glycosaminoglycan secretion. However, collagen formation by cultured fibroblasts was decreased (58, 59). The depressant effect on fibroblast numbers, previously noted with PHA-treated lymphocytes and PHA alone (58), may have obscured any enhanced accumulation of collagen, such as observed in the present report in the presence of appropriately diluted supernates of PHA-stimulated lymphocytes from which the PHA had been previously removed by immunoabsorption.

In another pertinent recent observation, Sisson et al. (60) showed stimulation of the production of glycosaminoglycan when retrobulbar fibroblasts were exposed to lymphocyte lysates. This activity was somewhat enhanced when the lymphocytes had been treated with PHA. However, lymphocyte supernates were not tested and collagen synthesis was not examined. The appearance of increased collagenous tissue in association with mononuclear infiltrates in retrobulbar connective tissue and muscle in Graves' disease (61) and in the liver in portal (62), postnecrotic (63), and biliary cirrhosis (64), and chronic hepatitis (64) are, however, well-documented phenomena.

Mitogens such as PHA are potent lymphocyte stimulators, causing cellular transformation and the release of a number of mediators of cellular immunity or lymphokines (65). In the studies reported here, lymphokine-rich supernates have been generated by PHA stimulation of normal human peripheral blood mononuclear cells. A direct inhibitory effect of PHA on collagen synthesis by fibroblasts was observed. This inhibition of collagen synthesis was obviated by immunoabsorptive removal of PHA from the supernates utilized. By suitable dilutions of these supernates, it was possible to show that enhanced collagen accumulation occurred when human embryonic lung (WI-38) fibroblasts were incubated with the lymphokine supernates. This activity was demonstrated in spite of the inhibitory lymphokine activities of lymphotoxin, proliferation inhibitory factor, and cloning inhibitory factor known to be present in such supernates.

Enhanced collagen accumulation was evidenced in three types of experiments. In the initial experiments the content of cell-associated and insoluble collagen in monolayers was assayed by a spectrophotometric analysis of hydroxyproline content. In a second type of assay, the incorporation of [$^{14}$C]proline into TCA-precipitable protein as [$^{14}$C]hydroxyproline was measured. In a third type, highly specific collagenase was utilized to determine the amounts of [$^{14}$C]proline and [$^{14}$C]hydroxyproline solubilized from the TCA precipitates of monolayers and supernates of fibroblast cultures incubated with PHA-stimulated mononuclear supernates. In all three types of experiments, enhanced collagen accumulation was observed in the presence of PHA-stimulated supernates compared with lymphocyte cultures to which PHA had not been added.

The last type of experiment, in which the amount of [$^{14}$C]proline and [$^{14}$C]hydroxyproline was measured in collagenase-solubilized protein, demonstrated that the increased collagen accumulation demonstrated in the first two types of experiments did not depend simply upon increased hydroxylation of newly synthesized collagen by fibroblasts treated with PHA-stimulated mononuclear cell supernates. Also, since increased collagen synthesis was demonstrated in the monolayer-insoluble fraction as well as in the culture supernate by both the [$^{14}$C]proline incorporation method and the collagenase digestion method, the possibility was eliminated that lymphokine-rich supernate simply changed the fractional distribution of supernate and monolayer-associated collagen by increased release of soluble collagen into the supernates. An alternative possibility, that the lymphokine-rich supernates decreased degradation of collagen, thereby apparently enhancing synthesis, was also eliminated by an experiment in which WI-38 fibroblasts were prelabeled with [$^{14}$C]proline and then exposed to both control and PHA-stimulated mononuclear cell supernates. In this experiment neither the lymphokine-containing nor the control supernate influenced the content of [$^{14}$C]-hydroxyproline-prelabeled, TCA-precipitable collagen present at the end of the exposure period.

Although supernates from some PHA-stimulated donor lymphoid cells appeared not to enhance collagen accumulation when results were expressed in absolute amounts per culture tube, they did appear to enhance accumulation when results were corrected for the number of viable fibroblasts present during the labeling period. In the experiment presented in Table XI, enhanced accumulation of monolayer collagen was observed only when correction was made for the number of surviving fibroblasts, although enhanced accumulation was noted in the supernates on an absolute basis. Since collagen accumulation in the latter experiments was measured by adding [$^{14}$C]proline after 44 h of culture and labeling during the subsequent 4-h interval, it would appear that correction in this case for the number of cells surviving at the end of 48 h was not subject to error introduced by the cytotoxic action of lymphotoxin during the labeling period, since the action of this lymphokine is largely complete by 30 h (44). This experiment sug-

gests that the enhanced collagen synthesis observed was the product of selected surviving fibroblasts.

LeRoy has recently reported (66) that fibroblasts isolated from scleroderma skin lesions produced greater quantities of collagen than isolates from normal skin. This finding is not incompatible with the possibility that lymphokines may select from a normal heterogenous population of fibroblasts a subpopulation that produces increased amounts of collagen.

The factor in the lymphokine-containing supernates that produced enhanced collagen accumulation was nondialyzable. It was produced in the absence of serum in the stimulating medium and was stable upon lyophilization and storage at − 70°C in either 1% Carbowax 6000 (donor 1 and donar 3 preparations) or 0.2% albumin (donor 2). It was detectable in dilutions of PHA-stimulated mononuclear supernates as high as 1 : 16 and was produced by purified populations of peripheral blood mononuclear cells consisting of lymphocytes and monocytes. This activity, for which we suggest the name fibroblast-stimulating activity, appears to have important implications in the development of fibrosis consequent to chronic inflammation. It may be especially significant in the fibrosis of PSS.

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