Mononuclear Cell Modulation of Connective Tissue Function

SUPPRESSION OF FIBROBLAST GROWTH BY STIMULATION OF ENDOGENOUS PROSTAGLANDIN PRODUCTION

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ABSTRACT The role of immune cell products in modulating connective tissue metabolism was investigated. Supernates of both unstimulated and phytohemagglutinin-stimulated human mononuclear cell cultures suppressed fibroblast proliferation (up to 90%) and concomitantly stimulated fibroblast prostaglandin E (PGE) synthesis (20- to 70-fold). The growth suppression was, at least in part, a secondary result of the increased fibroblast PGE synthesis; growth suppression (*a*) paralleled the increased fibroblast PGE synthesis, (*b*) was reversed by addition of inhibitors of prostaglandin synthesis (indomethacin, meclofenamate, and eicosatetraynoic acid), and (*c*) was reproduced by addition of exogenous PGE_2 to fibroblast cultures. The prostaglandin-stimulatory, growth-suppressive activity was a product of non-T-lymphocyte, adherent cells and was present within 6 h of mononuclear cell culture. The activity was heat (56°C) and trypsin sensitive, nondialyzable, and appeared in the 12,000–20,000 mol wt fractions on Sephadex G-100 chromatography. The activity in supernates of mononuclear cell cultures was removed by incubation with fibroblasts but not by similar incubation with peripheral blood mononuclear cells. Mononuclear cells release a factor(s) which modulates fibroblast proliferation by altering prostaglandin metabolism.

INTRODUCTION

Inflammatory lesions are characterized by the participation and interaction of several different cell types. One example is the cooperation of lymphocytes and monocytes in immune responses, an interaction mediated in part by release of soluble factors (lymphokines and monokines) which affect cell metabolism. The relationship between these mononuclear cells and other cell types involved in complex inflammatory states is less well defined.

The involvement of connective tissue elements in lesions such as granulomas, neoplasms, healing wounds, and vasculitis has generated interest in the modulation of connective tissue metabolism by immunologically active cell populations (1–6). Experimental evidence indicates that factors released by mononuclear cells (lymphocytes and monocytes) may affect fibroblast chemotaxis (1), stimulate fibroblast proliferation (2), and enhance collagen and proteoglycan synthesis (3, 4). The interaction between fibroblasts and mononuclear cells may be bidirectional; under certain conditions fibroblasts may produce migration inhibitory factor (7) and substitute for adherent cells in the immune response (8). Furthermore, connective tissue cell products, such as prostaglandins, may play a role in modulating immune function (9).

In the present studies, we investigated the ability of mononuclear cells to influence fibroblast proliferation. Supernates of human mononuclear cell cultures suppressed the proliferation of dermal fibroblasts in vitro. This suppression was mediated, at least in part, by stimulation of fibroblast prostaglandin synthesis.

METHODS

Cell cultures. Human dermal fibroblasts (FB)[1] obtained by collagenase digestion of normal neonatal foreskin were kindly provided by Dr. G. Sherer, Medical University of

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[1] *Abbreviations used in this paper*: E-RFC, cells forming rosettes with sheep erythrocytes; FB, fibroblasts; [^{3}H]TdR, tritium-labeled thymidine; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGB, PGE,

South Carolina. The cells had the typical spindle-shaped appearance of FB and synthesized collagen as 2–4% of cell protein. Cells were maintained in basal Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co.), 25 mM Hepes buffer, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were fed twice weekly, transferred at confluence, and used for experiments between the 8th and 30th subpassage in culture.

Supernatant preparation. Normal human peripheral blood mononuclear cells (PBMC) were isolated by flotation on Lymphocyte Separation Media (Litton Bionetics, Kensington, Md.) and washed three times in Hanks' balanced salt solution. For production of supernates (SN), cells (5×10^5/ml) were cultured for 72 h at 37°C in a 5% CO_2-humidified atmosphere in RPMI-1640 (Grand Island Biological Co.) supplemented with 20% heat-inactivated human AB serum, 25 mM Hepes buffer, 2 mM glutamine, penicillin, and streptomycin in 75-mm polypropylene tubes (Falcon Labware, Oxnard, Calif.) with or without phytohemagglutinin (Burroughs Wellcome, Research Triangle Park, N. C.), 2.5 μg/ml, the previously determined optimal mitogenic dose. To terminate cultures, tubes were centrifuged at 400 *g* for 10 min, and the SN medium was aspirated and passed through a 0.45-μm filter (Millipore, Corp., Bedford, Mass.). Cell-free SN were stored at −20°C and diluted in RPMI-1640 with 20% heat-inactivated AB serum before use (usually within 2 wk).

In some experiments, PBMC were fractionated before culture for SN preparation. Cell populations enriched and depleted of T lymphocytes were obtained by separation of cells forming rosettes with sheep erythrocytes (E-RFC) on a Ficoll-Hypaque gradient (10). Cells pelleting as E rosettes were 98% small lymphocytes (determined by examination of stained cytocentrifuge preparations) and 1% latex phagocytic. Cells remaining at the interface were 50% latex phagocytic and contained <5% E-RFC. Cells at the interface (non-E-RFC) were washed three times in RPMI-1640 and resuspended to 5×10^5 cells/ml in RPMI-1640/20% human AB serum. The pelleted cells (E-RFC) were washed once in RPMI-1640, resuspended in Tris-buffer, 0.81% NH_4Cl, pH 7.5 (9 parts NH_4Cl: 1 part buffer) to lyse the sheep erythrocytes and then washed three times in RPMI-1640 before resuspending at 5×10^5 cells/ml in RPMI-1640/20% AB serum.

PBMC were also depleted of adherent cells by incubating 5×10^6 cells in 5 ml of RPMI-1640/20% AB serum on 100-mm plastic dishes (Falcon 3003, Falcon Labware) for 2 h at 37°C in a 5% CO_2-humidified atmosphere. Nonadherent cells were carefully removed, washed once, and resuspended at 5×10^5 cells/ml in RPMI-1640/20% AB serum. Unfractionated cells and cells obtained after E-RFC separation or adherent cell depletion were then cultured as described above for preparation of SN.

Assay for SN activity. Confluent FB cultures were harvested by brief exposure to trypsin and resuspended (1×10^5 cells/ml) in RPMI-1640 with 20% human AB serum (identical batch of AB serum used for preparation of PBMC-SN). Equal 100-μl vol of the FB suspension and of diluted PBMC-SN or RPMI/20% AB serum were added to quadruplicate wells of flat-bottomed tissue culture plates (Microtest II, Falcon Labware) and cultured for 48 h at 37°C in a 5% CO_2-humidified atmosphere. 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol, Schwarz-Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.) was added to each well for the final 6 h of culture. At termination of the culture, medium was shaken off the plate, one drop of 0.25% trypsin (type III, Sigma Chemical Co., St. Louis, Mo.) with 0.02% EDTA was added to each well for 2 min and the cells were harvested on glass fiber filters (Reeve Angel, Clifton, N. J.) and washed with distilled water using a semiautomatic cell harvester (Otto Hiller Co., Madison, Wis.). Filter disks were counted in Aquasol-2 (New England Nuclear, Boston, Mass.) on a Beckman LS-345 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The standard deviation for quadruplicate samples was <10% and usually within 5% of the mean.

In some cases, parallel experiments for direct cell counts were performed in duplicate in 60-mm tissue culture dishes (Falcon Labware) using equal 1.5-ml vol of cell suspension (0.7×10^5 cells/ml) and PBMC-SN or RPMI/20% AB serum for each dish. Cultures were terminated at 48 h: the medium was aspirated and the cells treated with 0.25% trypsin-EDTA and resuspended in basal Eagle's medium-fetal bovine serum by vigorous pipetting to obtain a single cell suspension. Plates were examined microscopically to verify total cell removal. Cell counts were determined on a hemacytometer; each replicate plate was counted by two observers, and the results averaged.

Assay for prostaglandin production. Media of FB cultures were assayed for the presence of immunoreactive prostaglandin E-like material (iPGE). Cells were cultured as described above in quadruplicate wells of microtiter plates but were not pulsed with thymidine. At the termination of culture, 100 μl of medium was aspirated from each well; media from the first and second, and the third and fourth wells were pooled separately to yield duplicate 200-μl samples for iPGE assay. In some experiments, media (1 ml) of cultures performed in 60-mm tissue culture dishes were used for assay of iPGE. All assays were done on coded samples.

Radioimmunoassay of iPGE. [^{3}H]PGE_1 (1,500 cpm) (75 Ci/mM, New England Nuclear) and saline were added to aliquots of the media (0.15 or 1 ml) to bring the final volume to 3 ml. Samples were acidified to pH 3.5 with 9% formic acid and extracted twice with ice-cold ethyl acetate (12 ml). The extract was dried under a stream of nitrogen at 35°C, redissolved, and applied to a silicic acid column (0.5 g), and the PGE fraction collected (11). The PGE fraction was converted to PGB using 0.1 N KOH/methanol (12), and assayed using a previously described radioimmunoassay procedure (12). The antibody used cross-reacts with both prostaglandins E_1 and E_2 and therefore the term iPGE is used; the antibody does not cross-react significantly with the other major parent prostaglandins (11).

Absorption of SN activity. For absorption of SN activity, PBMC-SN were incubated with either FB or lymphocytes (10^7 cells/ml SN) for 1 h at 20°C followed by 1 h at 4°C. The mixture was then centrifuged (200 *g* for 10 min), and the SN was passed through a 0.45-μm filter. FB for absorption were obtained by 0.25% trypsin (type III, Sigma Chemical Co.)—0.02% EDTA treatment of confluent foreskin FB cultures. After trypsin treatment, FB were washed twice in RPMI-1640 and 10^7 pelleted cells resuspended in 1 ml of undiluted SN. PBMC for absorption, obtained by Lymphocyte Separation Media flotation of peripheral blood, were exposed for 3 min to the same trypsin-EDTA solution, washed twice in RPMI-1640, and resuspended in PBMC-SN (10^7 cells in 1 ml of SN). PBMC not exposed to trypsin were otherwise treated similarly and also used for absorption.

RESULTS

Suppression of FB growth by PBMC-SN. SN of both unstimulated and phytohemagglutinin (PHA)-

and PGF, prostaglandins B, E, and F; iPGE, immunoreactive prostaglandin E-like material; PHA, phytohemagglutinin; SN, supernate.

stimulated PBMC markedly suppressed tritium-labeled thymidine ([^{3}H]TdR) uptake by FB cultures. The suppression was dose dependent and present with all 60 SN preparations tested (Fig. 1). SN of PHA-stimulated cultures had greater suppressive activity than those of unstimulated cultures ($P < 0.01$ by Student's t test at all concentrations shown) although for some preparations the difference was apparent only at low SN concentrations. PHA alone, at concentrations of 0.02–2.5 μg/ml, had no effect on thymidine incorporation by FB (range 0–3, mean 1±2% [SEM] suppression).

Although suppression of cell number by SN preparations was usually evident by microscopic examination of the microtiter wells before harvesting, parallel experiments using direct cell counts were performed to insure that thymidine incorporation was a true reflection of cell proliferation. A suppression of cell number at the end of 48 h was evident in cultures containing either unstimulated or PHA-stimulated PBMC-SN (Table I). PHA alone (at a concentration equivalent to that present in the 25% PBMC-SN preparation, assuming no PHA had been consumed during the original PBMC culture) had no significant effect on cell number. Parallel experiments comparing [^{3}H]thymidine incorporation and direct cell counts showed a strong positive correlation ($n = 12, r = 0.89, P < 0.01$ by Student's t test).

The suppression of proliferation seen was not due to exhaustion of nutrients or lower effective serum concentration in the added SN. Addition of either 25% phosphate-buffered saline (PBS) or RPMI without serum instead of SN did not result in lowered [^{3}H]-TdR incorporation (not shown). PBMC-SN tested immediately after PBMC culture behaved similarly to stored frozen PBMC-SN. FB exposed to PBMC-SN remained viable (determined by nuclear exclusion of trypan blue) and were able to proliferate normally when PBMC-SN-containing medium was removed and replaced by RPMI-20% AB serum (not shown). Results obtained with four different foreskin FB strains were comparable. Similarly, there were no appreciable differences in results obtained using FB from the 8–12th compared with the 25–30th passage in culture.

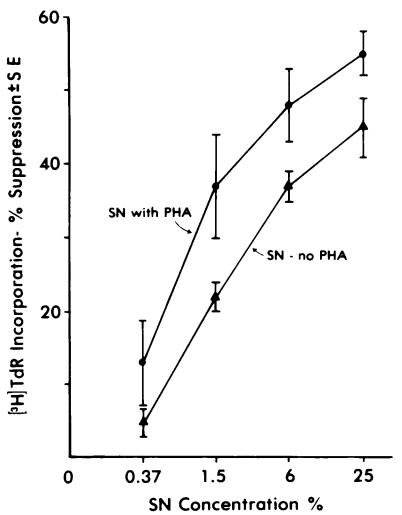

FIGURE 1 Effect of SN of PBMC cultures on FB proliferation. SN of unstimulated (SN with no PHA, $n = 21$) and PHA-stimulated (SN with PHA, $n = 39$) PBMC cultures from 25 different blood donors were tested over a 12-mo period for effect on FB [^{3}H]TdR incorporation. Results are expressed as percentage suppression of [^{3}H]TdR uptake (±SEM) compared with matched control cultures. [^{3}H]TdR uptake for control cultures ranged from 20,000 to 45,000 cpm in different experiments.

TABLE I
Suppression of Cell Proliferation by PBMC-SN

Addition	Cell numbers × 10^{-5}	Percentage suppression*
None	1.4±0.01‡	—
Unstimulated SN ($n = 4$)§	0.97±0.09	32±5‡
PHA-stimulated SN ($n = 4$)	0.72±0.06	49±5 $P < 0.05$‖
PHA, 0.62 μg/ml ($n = 3$)	1.4±0.05	0±5

In each experiment, 10^5 FB were plated on duplicate or triplicate 60-mm culture dishes in 3 ml of RPMI/20% AB serum containing no addition, 25% SN from unstimulated or PHA-stimulated PBMC (from the same PBMC donor), or PHA alone. Cells were counted following trypsinization after 48 h of culture.

* Percentage of suppression of cell number compared with cultures in RPMI/20% serum without additions.

‡ Mean±SEM.

§ n = number of experiments.

‖ Paired t test for stimulated vs. unstimulated SN.

Stimulation of prostaglandin synthesis in FB by PBMC-SN. FB grown in the presence of PBMC-SN showed a marked increase in the production of iPGE (Table II). Untreated FB cultures release little iPGE into the media. The addition of SN, either unstimulated or PHA-stimulated, resulted in a 50- to 70-fold increase in iPGE synthesis. Addition of PHA alone resulted in only a slight increase in iPGE production. The increase in iPGE in the cultures to which SN had been added could not be explained by passive transfer of iPGE from the mononuclear cell cultures since iPGE concentrations in SN preparations were only severalfold greater than that of media alone (3.3–4.7 vs. 0.7 ng/ml).

Relationship between prostaglandin synthesis and growth suppression. The increase in iPGE synthesis was dose dependent and paralleled the growth-suppressive effect at different PBMC-SN concentrations (Fig. 2). Both growth suppression and stimulation of prostaglandin synthesis were detectable within 6 h

TABLE II
Stimulation of FB iPGE Synthesis by PBMC-SN

	iPGE
	ng/ml
FB control	1.3*
FB + 25% unstimulated SN	55.4
FB + 25% PHA-stimulated SN	90.1
FB + PHA (0.62 μg/ml)	2.4
Media (RPMI/20% AB serum)	0.7
Unstimulated SN‡	3.3
PHA-stimulated SN‡	4.7

10^5 FB were plated in culture dishes as in Table I. After 48 h, 1 ml of medium was removed from each dish and frozen (−20°C) for iPGE assay.
* Mean of duplicate cultures.
‡ Tested undiluted.

after the addition of PBMC-SN and remained parallel for 48 h (Fig. 3). To investigate whether PGE played a role in the growth suppression, fibroblast cultures were treated with indomethacin, an inhibitor of prostaglandin synthesis. Indomethacin (1.0 μg/ml) added to FB cultures before the addition of PBMC-SN inhibited iPGE synthesis (Fig. 4). Concomitantly, there was inhibition of the SN-induced growth suppression, particularly evident with unstimulated SN at all concentrations and at lower concentrations of PHA-stimulated SN (Fig. 5). At high PHA-stimulated SN concentrations, only part of the growth-suppressive effect was reversed by the addition of indomethacin despite inhibition of iPGE synthesis, suggesting that some of the growth suppression occurred by a different mechanism. Indomethacin alone had no significant effect on FB growth.

A concentration of indomethacin (0.1 μg/ml) that inhibited the effect of dilute (1.5%) SN preparations often did not affect or only partially inhibited the effect of more concentrated (25%) PBMC-SN, whereas 1.0 μg/ml of indomethacin inhibited the effect of both SN concentrations (Table III). Addition of even higher

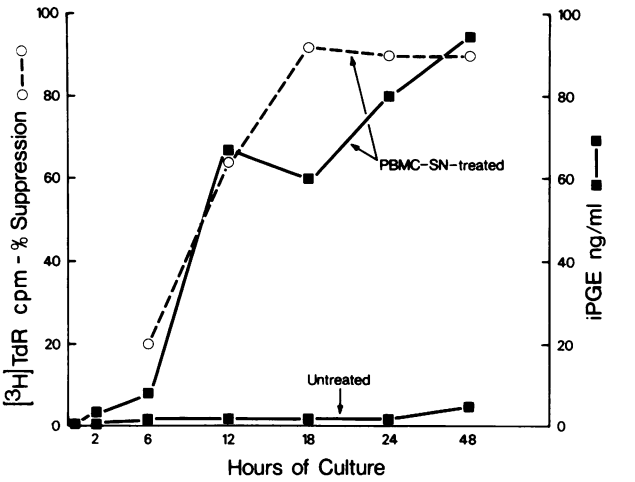

FIGURE 3 Time-course of suppression of FB growth and stimulation of iPGE synthesis by PBMC-SN. Replicate cultures were plated at 0 h with or without 25%-PBMC-SN (PHA stimulated). Quadruplicate wells were harvested at each time point shown (6 h after pulsing) for determination of [^{3}H]TdR incorporation. Separate quadruplicate wells were used for iPGE assay at each time point.

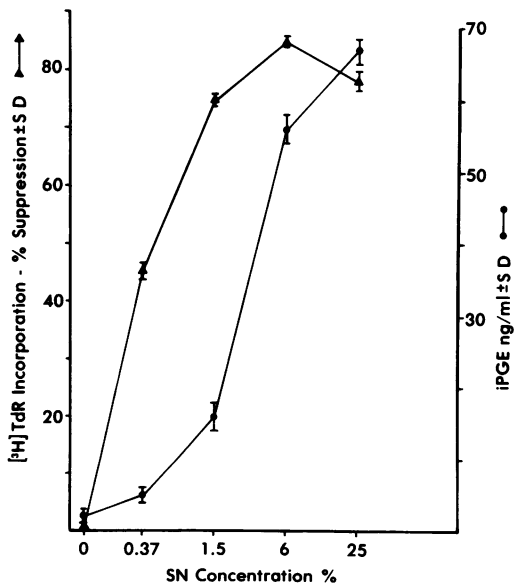

FIGURE 2 Suppression of [^{3}H]TdR incorporation and stimulation of PGE synthesis by PBMC-SN. Samples for [^{3}H]-TdR incorporation and iPGE synthesis were cultured simultaneously in microtiter plates using a single PHA-stimulated SN preparation. Each point is the mean±SD for quadruplicate ([^{3}H]TdR) or duplicate (iPGE) assays in a representative experiment.

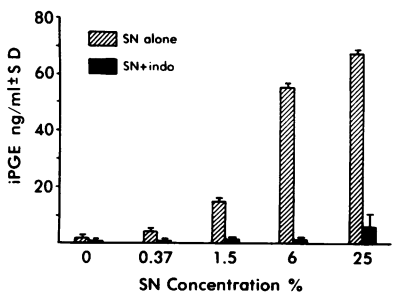

FIGURE 4 Inhibition by indomethacin of PBMC-SN-stimulated production of iPGE. Indomethacin (indo) (5 μl of a 40-μg/ml solution in 0.1 M, pH 7.8 phosphate buffer) was added to 10^4 FB in quadruplicate wells of microtiter plate; buffer alone was added to control wells. The cells were incubated for 2 h before the addition of PBMC-SN (PHA stimulated) and cultured for an additional 46 h. (Final indomethacin concentration 1 μg/ml.) Each bar represents the mean±SD of duplicate determinations.

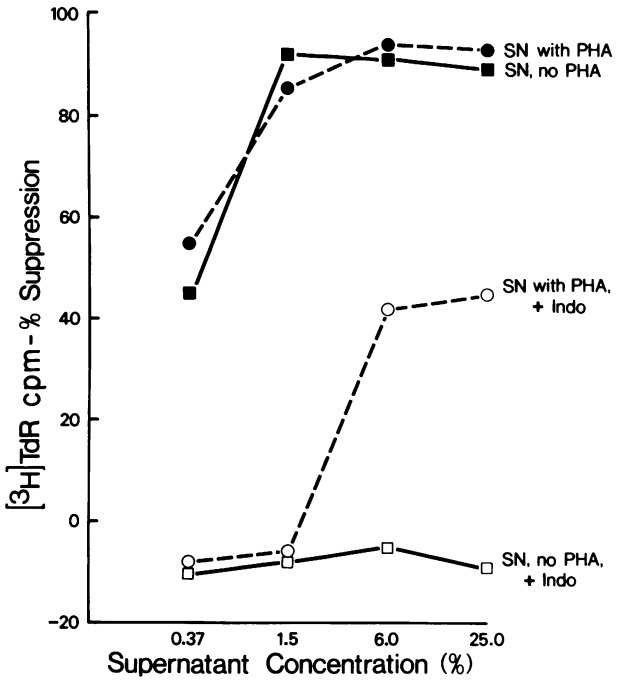

FIGURE 5 Reversal by indomethacin of PBMC-SN-induced growth suppression. FB were incubated with indomethacin (+Indo) or buffer, as previously noted, before addition of unstimulated or PHA-stimulated SN preparations. Both SN preparations were derived from PBMC obtained from a single donor on the same date. Indomethacin in the absence of PBMC-SN gave 1% suppression of [^{3}H]TdR incorporation.

concentrations of indomethacin (10 μg/ml) did not result in greater inhibition of suppression; however, this dose of indomethacin was frequently toxic to the cells, itself causing inhibition of thymidine incorporation (not shown).

Two other inhibitors of prostaglandin synthesis, sodium meclofenamate and eicosatetraynoic acid, similarly inhibited the PBMC-SN growth-suppressive effect without themselves affecting FB proliferation. The degree of inhibition depended on the dose of drug used (e.g., at 0.1, 1.0, and 10.0 μg/ml of eicosatetraynoic acid, 8, 31, and 73% inhibition of suppression, respectively).

Effect of PGE_2 on FB proliferation. To further examine the role of prostaglandins in inhibiting FB growth, PGE_2 was added to FB cultures (Table IV). Addition of 5–500 ng of PGE_2/ml of culture medium at the initiation of the culture had no significant effect on FB proliferation. Addition of 5,000 ng/ml resulted in a growth-suppressive effect similar to that seen with PBMC-SN. Because much lower concentrations of PGE were associated with growth suppression in PBMC-SN-treated cultures and because prostaglandins might be labile or rapidly metabolized, lower concentrations of PGE_2 were added at multiple time points

TABLE III
Inhibition of SN-induced Growth Suppression by Indomethacin

SN concentration	SN alone	SN + indomethacin 0.1 μg/ml	SN + indomethacin 1.0 μg/ml
%	[^{3}H]TdR cpm, % suppression		
0	0±4	(11)*±6	(8)±6
1.5	50±4	11±5	(4)±3
25	60±1	57±3	22±3

10^4 FB were seeded in replicate wells of microliter plates. Indomethacin was added 1 h later in a 5-μl vol to achieve the final concentration shown; phosphate buffer alone was added to wells not receiving indomethacin. SN or control medium (RMPI/20% AB serum) was added 2 h after indomethacin. Cultures were harvested at 48 h. Results are expressed as percentage suppression of [^{3}H]TdR incorporation (counts per minute) compared with cultures without SN or indomethacin. Each value represents the mean±SD for quadruplicate wells.

* Parentheses denote percentage stimulation.

during the culture period. The addition of 50 ng/ml of PGE_2 (the approximate level found in PBMC-SN-treated FB cultures, Fig. 2) at 0, 24, and 40 h of culture suppressed FB thymidine incorporation by 48% whereas a 10-fold higher concentration (500 ng/ml) added only at the beginning of the culture had no growth-suppressive effect (Table IV). The addition of exogenous PGE_2 was also able to reverse the inhibitory effect of indomethacin on PBMC-SN-induced growth suppression (Table IV). PBMC-SN thus suppressed thymidine incorporation by FB cultures, and this effect was reversed, in part, by the prior addition of indomethacin; addition of exogenous PGE_2 restored the growth suppression similar to that seen without indomethacin.

Production of growth-suppressive activity. To determine the cell of origin of the growth-suppressive and prostaglandin-stimulatory activities, PBMC were fractionated by depletion or enrichment of E-RFC (T lymphocytes) on a Ficoll-Hypaque gradient, and the resultant cell populations cultured for 72 h with PHA. SN of unfractionated PBMC and of PBMC depleted of E-RFC showed similar growth-suppressive activity (Table V). SN of E-RFC-enriched cultures caused little growth suppression. Media of FB cultures exposed to SN of unfractionated, E-RFC-enriched, and E-RFC-depleted PBMC cultures were assayed for iPGE. iPGE stimulatory activity was present in SN of both unfractionated and E-RFC-depleted cultures. SN of E-RFC-enriched PBMC gave almost no stimulation of iPGE synthesis (Table V). Similar experiments were done using PBMC depleted of adherent cells. SN of nonadherent PBMC did not suppress FB growth and did not stimulate iPGE synthesis (Table V). Thus, the

TABLE IV
Effect of PGE_2 on FB Growth

Addition	Time added	[³H]TdR incorporation, percentage suppression	Inhibition of suppression*
	h	*%*	*%*
Buffer	0	0±8	
PGE_2, *ng/ml*			
5	0	(11)‡±6	
50	0	(5)±5	
500	0	4±4	
5,000	0	52±3	
50	0, 24, 40	48±8	
500	0, 24, 40	72±5	
6% PBMC-SN		67±7	
6% PBMC-SN + indo		37±6	42
6% PBMC-SN + indo + PGE_2, *500 ng/ml*	0, 24, 40	62±7	7
Indo		8±2	

10^4 FB were seeded in replicate wells of microtiter plates at 0 h. Indomethacin (Indo) or buffer was added at 1 h in a 5-μl vol to achieve a final concentration of 1.0 μg/ml. PGE_2 diluted in 0.1 M phosphate buffer (or buffer alone) was added in 5-μl vol at times indicated. Concentration shown is final PGE_2 concentration in wells. SN (or control medium) was added to each well at 3 h of culture. Cultures were pulsed with [³H]TdR at 42 h and harvested at 48 h. Results are expressed as percentage suppression compared with control (medium plus buffer) wells. Each value represents the mean±SEM for two separate experiments.

* Inhibition of suppression by indomethacin calculated as: % suppression with PBMC-SN − (% suppression with PBMC-SN + Indo)/% suppression with PBMC-SN × 100.

‡ Values in parentheses denote stimulation.

TABLE V
Effect of Mononuclear Cell Subpopulations

	SN concentration percentage				
	0.37	1.5	6	25	iPGE
	[³H]TdR cpm, % suppression				*ng/ml*
SN source					
No SN	–	–	–	–	2.4±0.6
Unfractionated MC*	34	55	81	90	40.0±1.4
T-enriched MC*	13	(14)‡	(1)	19	3.8±0.2
T-depleted MC*	24	59	77	ND§	24.0±0.5
No SN	—	—	—	—	2.9±0.2
Unfractionated MC	2	60	95	97	53±5.3
Nonadherent MC	(2)	0	(4)	9	1.7±0.04

PHA-stimulated SN of various mononuclear cell (MC) populations were tested at concentrations shown for effect on FB [³H]TdR incorporation and iPGE synthesis. Each value for percentage suppression represents the mean of quadruplicate wells. Each value for iPGE is the mean (±SD) for duplicate determinations.

* Unstimulated and PHA-stimulated [³H]TdR incorporation, respectively, for the mononuclear cell fractions used to make SN were: unfractionated, 200 and 171,843 cpm; T-enriched MC, 128 and 20,465 cpm; and T-depleted MC, 411 and 2,210 cpm.

‡ Parentheses denote percentage stimulation.

§ Not determined.

growth-suppressive and prostaglandin-stimulatory activities both appeared in the same cell fraction.

PBMC isolated by Ficoll-Hypaque density gradient centrifugation contain variable numbers of contaminating platelets. To examine the possible role of contaminating platelets, PBMC were layered over fetal calf serum and centrifuged for 5 min at 150 *g*. With this procedure, >90% of the platelets remain suspended in the fetal calf serum. The pelleted mononuclear cells and the platelet-rich suspensions were washed and cultured separately for 72 h. Cell-free SN of the mononuclear cells and platelets were tested for effect on FB proliferation. SN (25%) of platelet suspensions did not affect FB proliferation (5% stimulation and 1% suppression of FB [³H]TdR incorporation by unstimulated and PHA-stimulated platelet SN, respectively). SN of unstimulated and PHA-stimulated mononuclear cells suppressed [³H]TdR incorporation by 49 and 91%, respectively. The lack of an effect of platelet SN is consistent with the lack of activity of nonadherent cells which would contain almost all the contaminating platelets.

Growth-suppressive activity appeared in the SN of PHA-stimulated PBMC cultures within 2 h (Table VI). The growth-suppressive activity in SN of 2-h cultures was present only at the highest concentration tested and was completely reversed by indomethacin. The growth-suppressive activity in SN of 6-, 24-, and 72-h cultures was evident at lower SN concentrations (i.e., present at higher titer); at higher SN concentrations, it was only partly reversed by prior addition of indomethacin. PGE-dependent, indomethacin-reversible growth-suppressive activity thus appears earlier in the SN of PBMC cultures and is present in higher titer than the prostaglandin-independent growth-suppressive activity.

Physical characteristics of PBMC-SN activity. The growth-suppressive activity in PBMC-SN was partially destroyed by heating at 56°C for 1 h and completely destroyed by similar incubation at 80°C (not shown). Incubation at 56°C resulted in a reduction of both indomethacin-reversible and indomethacin-irreversible

TABLE VI
Time-course for Appearance of PBMC-SN Activity

	SN concentration percentage			
	0.37	1.5	6	25
	[³H]TdR cpm, % suppression			
SN preparation				
2 h	2	(6)*	(11)	77
+ Indomethacin‡	(24)	(16)	(16)	(4)
6 h	49	96	98	95
+ Indomethacin‡	(6)	34	59	66
24 h	88	95	94	95
+ Indomethacin‡	(6)	38	45	57
72 h	64	90	92	91
+ Indomethacin‡	(5)	32	55	65

PBMC were cultured in RPM/20% AB serum with 2.5 μg/ml PHA. Separate cultures were terminated at 2, 6, 24, and 72 h and SN was rendered cell free by passage through a 0.45-μm filter and stored at −20°C until use. Indomethacin and SN were added to replicate wells of 10^4 FB as in Table II. Each value is the mean of quadruplicate cultures.
* Parentheses denote percentage stimulation.
‡ Final indomethacin concentration, 1.0 μg/ml.

TABLE VII
Trypsin Treatment of PBMC-SN

Addition	SN concentration percentage			
	0.19	0.75	3.1	12.5
	[³H]TdR cpm, % suppression			
PBMC-SN + PBS	8	31‡	67	80‡
+ Indomethacin*		12		57
PBMC-SN + T-STI	16	17‡	13‡	33‡
+ indomethacin		11		26
RPMI/20% AB + T-STI	10	12	14	24
+ indomethacin		11		24

1 ml of SN was incubated for 1 h at 37°C with 0.5 ml of 1% trypsin (T) (type III, Sigma Chemical Co.) in PBS; 0.5 ml of 1% soybean trypsin inhibitor (STI) (type I-S, Sigma Chemical Co.) in PBS was then added for 10 min. Controls consisted of RPMI 1640/20% AB serum similarly treated and SN incubated similarly with PBS. All dilutions were made in 50% RPMI-AB serum/50% PBS. Final concentration of serum in all wells was 15% and of PBS, 25%. [³H]TdR incorporation (percentage suppression) is compared with FB cultured in RPMI/15% AB serum/25% PBS. Each value is the mean of quadruplicate cultures.
* Final indomethacin concentration 1.0 μg/ml.
‡ $P < 0.01$ for comparisons shown (Student's t test).

growth suppression, both of which could be detected only at high SN concentrations.

PBMC-SN and control media (RPMI/20% AB) were treated with trypsin and soybean trypsin inhibitor or with PBS and tested for effect on FB proliferation (Table VII). Control media treated with trypsin-trypsin-inhibitor suppressed FB thymidine incorporation slightly when compared with PBS-treated media. The growth-suppressive activity of PBMC-SN was almost completely abolished by trypsin treatment; residual growth suppression approximated that seen with trypsin-trypsin inhibitor-treated control media and was not reversed by indomethacin (Table VII).

PBMC-SN was fractionated by molecular sieve chromatography on G-100 Sephadex (Pharmacia Fine Chemicals, Div. Pharmacia Inc., Piscataway, N. J.). Column fractions were pooled (Fig. 6A), dialyzed, lyophilized, reconstituted in RPMI-1640, and tested for their effect on FB proliferation and iPGE synthesis. Control medium (RPMI/20% AB serum) was treated in an identical manner. When pooled column fractions were tested, growth-suppressive activity appeared in fraction III and, to a much lesser extent, in fraction I of chromatographed SN but not in the comparable control fractions (Fig. 6B). The activities of untreated SN and control medium and of unfractionated, but dialyzed, lyophilized material are shown for comparison; dialysis and lyophilization had no significant effect on SN-mediated suppression of FB proliferation. The same column fractions of SN and control medium were tested for their effect on FB iPGE synthesis (Fig. 6C). FB iPGE synthesis in untreated control medium was 1.2±0.1 ng/ml. There was a 40- to 50-fold stimulation of iPGE synthesis by PBMC-SN which was unaffected by dialysis of the SN. In chromatographed SN, prostaglandin-stimulatory activity was present largely in fraction III and to a lesser extent in fraction I, the same fractions that suppressed FB [³H]TdR incorporation. A small increase in iPGE synthesis occurred with dialyzed, lyophilized control medium, control fraction I, and SN fraction II. For all values shown in Fig. 6, there was a positive correlation between suppression of [³H]TdR incorporation and stimulation of iPGE synthesis ($r = 0.94$). Since dialysis (exclusion ~12,000 mol wt) did not result in any appreciable loss of activity, the active factor(s) in PBMC-SN preparations appears to have a molecular weight of 12,000–20,000. A smaller peak of activity present in the void volume may represent binding of the lower molecular weight material to larger proteins.

Absorption of PBMC-SN activity. PBMC-SN was incubated with FB to determine whether the growth-suppressive, prostaglandin-stimulatory activity could be removed. PBMC and trypsin-treated PBMC were used as absorption controls, the latter because FB used for absorption were trypsinized. Absorption of PBMC-SN with FB removed most of the growth-suppressive

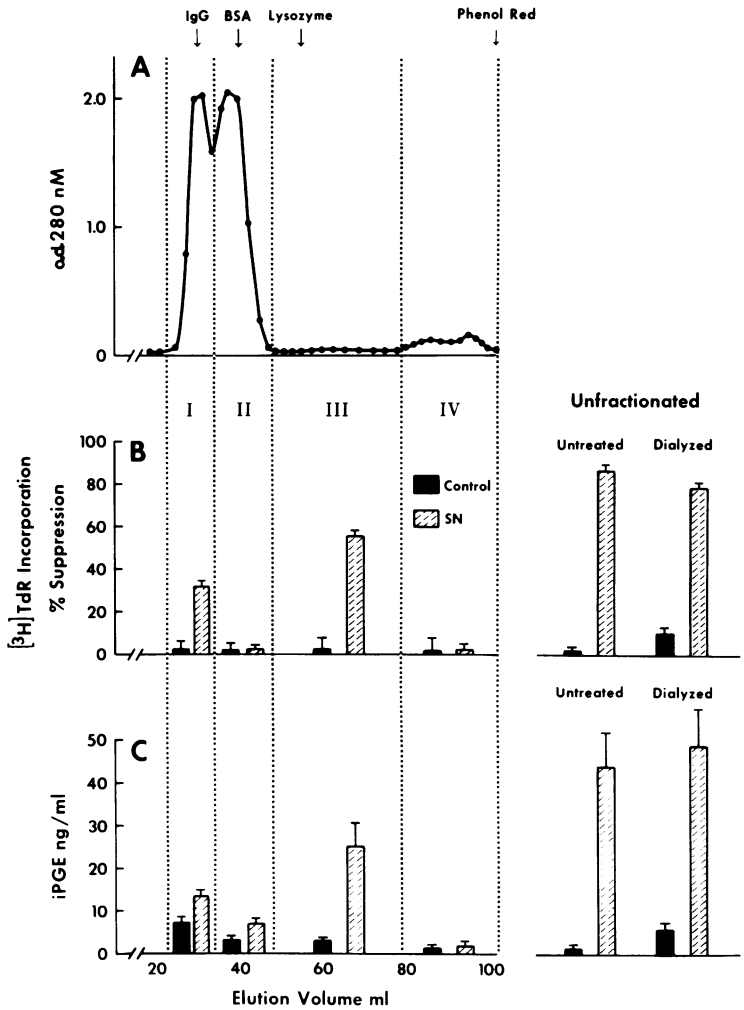

FIGURE 6 Column fractionation of PBMC-SN. (A) PBMC-SN was chromatographed on a calibrated Sephadex G-100 column with PBS (with 0.02% sodium azide) as the eluting buffer. Marker proteins shown are human IgG (150,000 mol wt), bovine serum albumin (BSA, 66,500 mol wt), and lysozyme (14,600 mol wt). Column fractions were pooled as shown (I-IV), dialyzed twice for 24 h against 500 vol of distilled water, lyophilized, and reconstituted to the original sample volume in RPMI-1640 without serum. A sample of RPMI-1640/20% AB serum (control medium) was treated in an identical manner; the elution profile for control medium was similar to that of PBMC-SN and comparable pooled fractions (I-IV) were prepared. (B) Fractions I-IV of PBMC-SN (hatched bars) and control medium (solid bars) were tested for effect on FB [^{3}H]TdR incorporation. Bars indicate percentage suppression of [^{3}H]TdR incorporation ±SD (compared with untreated control medium); each pooled fraction was tested at a 25% final concentration. Activity of untreated control medium and PBMC-SN as well as nonchromatographed but dialyzed and lyophilized control medium and PBMC-SN are shown on the right of the panel. (C) Fractions I-IV were similarly tested for effect on FB iPGE synthesis. Activities of untreated and of dialyzed, lyophilized but nonchromatographed control medium and PBMC-SN are shown on the right of the panel.

activity; a 16-fold greater SN concentration was needed for comparable suppression of [^{3}H]TdR uptake after absorption (Table VIII). Absorption with either untreated or trypsin-treated PBMC did not affect the activity of SN preparations. Similarly, FB absorbed SN preparations gave less stimulation of iPGE synthesis than did unabsorbed or lymphocyte-absorbed SN (Table VIII).

PHA-stimulated PBMC-SN was then tested for effect on thymidine incorporation by fresh PHA-stimulated lymphocyte cultures (Table IX). Addition of PBMC-SN to lymphocyte cultures stimulated [^{3}H]TdR uptake

TABLE VIII
Absorption of SN Growth-Suppressive Activity

SN treatment	SN concentration 0.37	1.5	6	25	iPGE
	[³H]TdR cpm, % suppression				ng/ml*
Unabsorbed	17	55	91	94	70
FB-absorbed	(2)‡	(6)	27	60	28
Lymphocyte absorbed§	1	43	87	90	78
Lymphocyte absorbed	10	48	89	95	87

SN were incubated with either FB or lymphocytes (10^7 cells/ml of SN) before assay. Experiments were performed in replicate microtiter wells and each value for [³H]TdR incorporation is the mean of quadruplicate wells.
* FB were cultured with 25% PBMC-SN for determination of iPGE synthesis. FB cultured without added SN synthesized 2.5±0.1 ng/ml of iPGE. Each value is the mean of duplicate samples.
‡ Values in parentheses denote stimulation.
§ Lymphocytes treated with trypsin before being used for absorption.

in response to PHA. The same PBMC-SN suppressed FB [³H]TdR incorporation. The failure of PBMC-SN to suppress lymphocyte-proliferative responses correlates with the inability of these cells to absorb growth-suppressive activity from PBMC-SN.

DISCUSSION

Mononuclear cells produce a variety of cytokines, some of which may affect nonlymphoid, nonmonocyte cell populations (13–17). The data presented show that SN of unstimulated as well as PHA-stimulated mononuclear cell cultures suppress in vitro proliferation of human dermal FB. Two types of growth suppression were effected by PBMC-SN. With SN of unstimulated PBMC and at lower SN concentrations of PHA-stimulated PBMC, the suppression of proliferation was due, in large part, to stimulation of FB prostaglandin synthesis because it was reversed by indomethacin and reconstituted by exogenous PGE_2. This prostaglandin-dependent suppressive activity appears to reside in a peptide product(s) of adherent mononuclear cells. At higher concentrations of PHA-stimulated PBMC-SN, the growth suppression was often not completely indomethacin reversible, despite inhibition of PGE synthesis, indicating the presence of (an) additional growth-suppressive factor(s). The increased suppression effected by SN of PHA-stimulated PBMC compared with SN of unstimulated PBMC may result from such additional growth-supressive factors.

TABLE IX
Effect of PBMC-SN on Lymphocytes

Target cell	SN concentration 0.37	1.5	6	25
	[³H]TdR cpm, % suppression			
Lymphocytes*	(1)‡	(32)	(40)	(36)
Fibroblasts*	15	25	57	67

PBMC-SN was tested simultaneously for effect on FB and lymphocyte proliferation. FB were cultured at 10^4 cells/well and lymphocytes at 10^5 cells/well; PHA, 2.5 μg/ml, was added to the lymphocyte cultures.
* Counts per minute for non-SN-treated cultures were 36,253 ±2,662, and 95,569±2,006 for FB and lymphocytes, respectively.
‡ Values in parentheses denote percentage stimulation.

Mononuclear cells isolated from heparinized blood are contaminated by platelets and a contributing role of these platelets to the PBMC-SN activity must be considered. However, the absence of activity in SN of platelet-rich suspensions and the loss of activity with removal of adherent cells makes it unlikely that platelets have a significant role in the elaboration of PBMC-SN activity. In addition, we have prepared FB growth-suppressive, prostaglandin-stimulatory SN using platelet-free mononuclear cells isolated from rheumatoid synovial tissue (18) indicating that the activity is, indeed, mononuclear cell derived.

A number of factors have been identified in SN of mononuclear cell cultures which may suppress cell proliferation. Lymphotoxin, a T-lymphocyte product, suppresses cell proliferation by a cytotoxic effect (13). Proliferation inhibitory factor can be detected in SN of unstimulated mononuclear cells and suppresses growth of a variety of target cells (14); proliferation inhibitory factor may be identical to lymphotoxin, a noncytotoxic but growth-suppressive activity resulting at low concentrations of proliferation inhibitory factor-lymphotoxin (19, 20). Inhibitor of DNA synthesis, an 80,000 mol wt glycoprotein product of rat lymphocytes, can suppress proliferation of FB as well as of mitogen-stimulated lymphocytes (14). The mechanism of inhibitor of DNA synthesis action appears to be stimulation of cAMP in the target cell (21, 22). Since PGE can stimulate cAMP synthesis, the factor(s) responsible for the indomethacin-reversible growth suppression observed in the present report might be related to inhibitor of DNA synthesis. Unlike inhibitor of DNA synthesis, our PBMC-SN does not suppress PHA-induced lymphocyte proliferation, does not appear to be produced by T lymphocytes, and is of considerably lower molecular weight.

The high level of PGE_2 synthesis by PBMC-SN-stimulated FB (~10 ng/10^4 cells in 12 h, Fig. 3) is similar to the levels reported for synovial cells from pa-

tients with rheumatoid arthritis (23), cells that presumably are subject to in vivo activation. Furthermore, synovial cells were shown to increase PGE_2 synthesis in response to a 10,000–20,000 mol wt factor released by mitogen-activated mononuclear cells (6); the increased PGE_2 synthesis was accompanied by a mild suppression of proliferation. Human lymphoblastoid and human FB interferon, as well as the interferon inducer polyinosinate·polycyctidylate [poly(I)·poly(C)] have also been shown to stimulate PGE production by synovial FB (24). Interferon suppressed FB proliferation in addition to stimulating PGE synthesis. Mononuclear cell SN could contain both interferon and interferon-inducing activity. Conversely, Wahl et al. (25) found that SN of guinea pig T lymphocytes stimulated both proliferation and PGE_2 synthesis of isologous fibroblasts. No investigation was made of the role of the increased PGE_2 synthesis in altering cell proliferation. Similarly, CTAP-III, a factor derived from an extract of human platelets, has been shown to stimulate both FB PGE synthesis and FB proliferation (26, 27). A growth-suppressive effect of PGE in these studies could have been masked by the presence of direct growth-stimulatory activity.

Prostaglandins can affect growth and function of FB in culture. Prostaglandin F_2 has been shown to initiate cell proliferation in mouse FB (28). Prostaglandins of the E series, conversely, have been shown to suppress cell proliferation (29). The effect of prostaglandins on FB may depend not only on the type of prostaglandin, but also on the FB population employed, the state of endogenous prostaglandin and cAMP production, and the age of the culture (29, 30). Thus, inhibitors of prostaglandin synthesis were shown to have different effects on FB proliferation depending on the point in time when they were added to cultures (30). Furthermore, even within an apparently homogeneous cell population there may be a heterogeneity of sensitivity to prostaglandin effects (29). In our studies, prostaglandins of the E series were responsible for at least part of the growth suppression; production of iPGE by the FB was demonstrated, and addition of exogenous PGE_2 duplicated the growth suppression. A contribution of prostaglandins other than PGE_2 to the indomethacin-reversible growth suppression cannot be excluded.

Prostaglandins may affect a diversity of cell functions in addition to cellular proliferation. In studies of rheumatoid synovial tissue, Dayer et al. (31, 32) found that PGE_2 may modulate both collagen synthesis and the secretion of latent collagenase (32) by adherent rheumatoid synovial cells. The interferon-induced stimulation of synovial cell hyaluronic acid synthesis also appears to be mediated, in part, by PGE_2 (33). These effects may be secondary to PGE_2-induced increases in cellular cAMP levels (34).

The absorption of PBMC-SN activity by FB suggests the presence of a cell surface receptor for the PGE-stimulatory product(s). The findings that PHA-induced lymphocyte proliferation was unaffected by PBMC-SN might be due to absence of such a receptor on lymphocytes, an explanation supported by the failure of these cells to absorb the PBMC-SN activity. It is possible, however, that continued release of new, active SN during the absorption incubation might obscure any loss of activity.

The induction of a prostaglandin-mediated autoregulatory phenomenon in FB by lymphocyte/monocyte products could be important in modulation of immune function. Several studies have indicated that prostaglandins may affect immunologically active cell populations. Adherent cells can suppress human T-cell mitogenesis by liberating prostaglandins (9). Tumor cells have similarly been shown to suppress immune responsiveness by producing prostaglandins (35). Macrophage response to endotoxin is mediated, at least partially, by prostaglandins: the endotoxin-stimulated synthesis of collagenase is dependent upon prostaglandin synthesis (36). Exogenous PGE_2 also stimulates macrophage collagenase production. Thus, fibroblasts can not only regulate their own growth by producing prostaglandins, as shown in the present study, but may also affect the activity of other cell types.

These potential interactions between connective tissue cells and immune cells may be important in the evolution of inflammatory lesions. The transition from inflammation and immune reactivity to healing and scar formation may be governed by signals between inflammatory cells and connective tissue cells. Disturbance of normal interactions could play a role in the pathogenesis of chronic inflammatory and fibrotic states. For example, chronic exposure of connective tissue cells to immune cell products might allow the selective and, perhaps unbridled, proliferation of FB populations. These, in turn, might prove resistant to "normal" immunologically mediated growth suppression or might have metabolic abnormalities that promote disease (23, 37–38).

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