INHIBITION OF COLLAGEN SYNTHESIS BY
MONONUCLEAR CELL SUPERNATES*

By SERGIO A. JIMENEZ,‡ WILLIAM McARTHUR,§ AND JOEL ROSENBLOOM

From the Department of Medicine, School of Medicine, and Center for Oral Health Research, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The involvement of various connective tissue components in the development of chronic inflammatory reactions has been noted for a long time. Early pathological descriptions identified fibroblasts and collagen as important components of such reactions (For review see Reference 1). The mechanisms responsible for the regulation of the connective tissue response, however, have not been clearly elucidated. The mitogenic effect on fibroblasts and the stimulation of their biosynthetic capacity by serum and other humoral components have only recently been demonstrated (2–5). Other factors capable of fibroblast stimulation have also been identified (For review, see Reference 6) but have not been studied in detail. The concept that a substance elaborated by inflammatory cells may stimulate fibrogenesis was derived from the finding of fibroblasts and collagen in close proximity to inflammatory cells, particularly monocytes, at sites of repair or chronic inflammation. Early experimental evidence to support this notion was obtained by Carrel who demonstrated that depletion of granulocytes resulted in delayed and faulty fibrogenesis (7). Subsequent work by many investigators, however, has yielded inconclusive and sometimes contradictory results (8–14). During development of chronic inflammatory reactions the involvement of the connective tissue is self-limited and under normal conditions fibroblast proliferation and synthetic activity cease when adequate repair has been achieved (15). These findings suggest that there must be sensitive mechanisms capable of regulating the duration and extent of fibroblastic activity which terminate the connective tissue involvement at some point in the course of the inflammatory reaction. If such mechanisms are deficient or faulty, excessive fibroblast proliferation and accumulation of their biosynthetic products may occur. In this paper we report the identification of a factor(s) released from human mononuclear cells which produces a selective inhibition of collagen synthesis when added to normal human skin fibroblasts in monolayer cultures.

Materials and Methods

Isolation of Mononuclear Cells and Generation of Culture Supernates. Human mononuclear cell suspensions were obtained from heparinized (10 IU/ml) peripheral blood obtained by venipuncture from young adults. Mononuclear cells were separated from the granulocytes and

erythrocytes by centrifugation (400 g) of the whole blood over a lymphocyte separation medium (LSM[1] solution, a Ficoll preparation supplied by Litton Bionetics, Kensington, Md.) for 30 min at 22°C (16). Mononuclear lymphocytes and monocytes were recovered at the LSM plasma interface and washed twice with Hank's balanced salt solution by centrifugation. Greater than 98% of the mononuclear cells were viable as measured by exclusion of trypan blue in a hemocytometer. Cells were suspended to a final concentration of 1-2 × 10⁶ cells/ml in RPMI-1640 medium supplemented with glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) and incubated at 37°C for 72 h in an atmosphere of 5% CO_2 and 95% air (17). Phytohemagglutinin -P (PHA), (Difco Laboratories, Detroit, Mich.), 1 µl/ml, was added to one-half of the mononuclear cell cultures. At the end of the incubation, cells and media were separated by centrifugation and the media exhaustively dialyzed against Hank's balanced salt solution. The preparations remained active for months when stored at −85°C. To test the mitogenic response of the mononuclear cells, samples from triplicate cultures of both PHA-stimulated and controls received [³H]thymidine (0.5 µCi/ml, New England Nuclear, Boston, Mass.) 16 h before the termination of the incubation period. The degree of incorporation of [³H]thymidine in the cultures was assessed at the end of the incubation period by collecting the cells on 25-mm glass-fiber filters, rinsing them with physiologic saline, and counting the dried filters in a liquid scintillation counter. The stimulation index is defined as the ratio of [³H]thymidine incorporation in PHA-containing cultures compared to control cultures. Only cultures having a stimulation index of at least 10 were used as a source of stimulated supernate.

Growth of Cells. Human fibroblasts were isolated from skin biopsies from healthy volunteers (18). The cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum in an atmosphere of 5% CO_2. The cells were used between passages 10 and 15. Routinely, the cells were plated at a density of 19,000/cm² in 30-mm plastic culture dishes and 5 d later when they were confluent, the experiments with the mononuclear preparations were begun. The media were changed and various dilutions of the mononuclear preparations with 1% fetal calf serum and 50 µg/ml ascorbate in Eagle's minimal essential medium were added, generally for 2 d. The media were then again replaced with media containing the same additions but with 0.5 to 2 µCi/ml [¹⁴C]proline (New England Nuclear), and 100 µg/ml β-aminoproprionitrile, and the incubation continued for 24 h.

Analyses of Labeled Proteins. At the end of the 24-h incubation with the [¹⁴C]proline, the media were removed and treated in one of two ways. In most experiments, the following reagents were added to the following final concentrations: sodium dodecyl sulfate, 1%; mercaptoethanol, 1%; EDTA, 4 mM; and paramethylsulfonyl fluoride, 10 µg/ml. The media were placed in a boiling water bath for 2 min and then dialyzed against 0.01 M sodium phosphate, pH 7.4, 0.1% mercaptoethanol and 0.1% sodium dodecyl sulfate. In the experiments involving digestion with bacterial collagenase, EDTA and paramethylsulfonyl fluoride were added to the media and they were then dialyzed against 0.05 M Tris·HCl, pH 7.4, 0.15 M NaCl. The monolayers containing cells and adherent protein were dissolved in 1% sodium dodecyl sulfate containing 4 mM EDTA and 10 µg/ml paramethylsulfonyl fluoride and the solutions heated and dialyzed as described for the media.

Collagenase digestion was performed with bacterial enzyme purified by DEAE chromatography and isoelectric focusing of Worthington highly purified bacterial collagenase (19) (Worthington Biochemical Corp., Freehold, N.J.). It had no nonspecific proteolytic activity when tested against various noncollagenous substrates. In these experiments, the 1-ml test samples were digested for 6 h at 37°C with 30 µg/ml enzyme in 0.05 M Tris-HCl, pH 7.4, 5 mM $CaCl_2$, and 0.15 M NaCl. The incubation was terminated by the addition of EDTA to a final concentration of 10 mM and sodium dodecyl sulfate and mercaptoethanol to 1%. The samples were placed in a boiling water bath for 2 min, and then dialyzed against two changes of distilled water of 10 ml each. The dialyzates and retentates were analyzed for total ¹⁴C and [¹⁴C]hydroxyproline by the method of Juva and Prockop (20).

Chromatography of labeled proteins in sodium dodecyl sulfate was performed on 1.5 × 85-cm columns of Agarose A-5m, 200 to 400 mesh (Bio-Rad Laboratories, Richmond, Calif.) as previously described (21).

---

[1] *Abbreviations used in this paper:* LSM, lymphocyte separation medium; PHA, phytohemagglutinin.

TABLE I

Effect of Fetal Calf Serum on Incorporation of [^{14}C]Proline and Synthesis of [^{14}C]-Hydroxyproline by Cultured Fibroblasts*

Serum concentration	Total ^{14}C	[^{14}C]Hyp	Degree of hydroxyl-ation‡
%	cpm × 10^{-3}	cpm × 10^{-3}	%
0	19.4	2.5	13.0
0.5	40.2	8.2	20.4
1.0	61.3	16.1	26.3
2.0	63.5	16.1	25.4
3.0	70.9	17.2	24.2
4.0	73.2	17.0	23.2
5.0	75.5	19.3	25.6
10.0	74.5	20.4	27.4

* Confluent fibroblast cultures were incubated with the concentrations of serum indicated and 50 μg/ml ascorbate for 48 h and then fresh media containing the same concentrations of serum and 1.0 μCi/ml [^{14}C]proline, 50 μg/ml ascorbate, and 100 μg/ml β-aminopropionitrile were added. After 24 h, the proteins secreted into the media were analyzed as described in Materials and Methods. The values represent the average of duplicate cultures, which agreed within 10% with respect to total incorporation and [^{14}C]hydroxyproline content.

‡ Values are [^{14}C]hydroxyproline per total ^{14}C × 100.

Results

Effects of Serum. In studies by other investigators testing the effects of mononuclear products on collagen synthesis, the fibroblasts under study have been incubated with the test preparations in variable concentrations of serum. These concentrations ranged from 0 to 10% (8, 13, 14). Our initial experiments with the mononuclear supernates were performed using serum-free medium and gave variable results. In some instances, stimulation of collagen synthesis was observed and in other instances inhibition was found relative to control cultures. This prompted an examination of the effect of serum on collagen synthesis by normal human fibroblasts. Table I demonstrates the dependence on serum concentration of the incorporation of [^{14}C]proline and the synthesis of [^{14}C]hydroxyproline in protein secreted by these cells. These experiments demonstrated that under these conditions using β-aminopropionitrile >85% of the [^{14}C]hydroxyproline was found in collagen secreted into the medium, and therefore the results with the cell layers are not shown. Other noncollagenous proteins containing [^{14}C]proline appeared also to be secreted. We found that both the total incorporation and the quantity of [^{14}C]hydroxyproline in the secreted proteins increased with increasing serum concentration. Whereas the maximum extent of hydroxylation was reached at 1% serum, incorporation continued to increase up to a serum concentration of 3–5%. Thus, experiments carried out in the absence of serum would most likely detect only stimulation of collagen synthesis, because in this condition, cells synthesize much smaller amounts of collagen than they do in 10% serum, the concentration most frequently used with fibroblasts in culture. We reasoned that 10% serum might mask an inhibitory effect of the added mononuclear supernates and we chose, therefore, to perform further experiments in 1% serum, a concentration which would probably permit detection of either stimulation or inhibition of collagen synthesis. All experiments were performed with confluent cultures to eliminate possible complicating effects of cell growth.

TABLE II

*Effect of Mononuclear Supernates on [^{14}C]Proline Incorporation and [^{14}C]-Hydroxyproline Synthesis**

Mononuclear supernatant addition	Total ^{14}C	[^{14}C]Hyp	Degree of hydroxyl-ation‡
	cpm × 10^{-3}	*cpm × 10^{-3}*	*%*
None	67.2	20.1	30.0
PHA-stimulated	69.9	1.9	2.7
Non-stimulated	72.0	8.2	11.4
Non-stimulated plus PHA§	69.4	8.2	11.8
PHA alone§	68.3	19.4	28.4

* Confluent fibroblast cultures were incubated with 400 µl of the additions listed in the table in a total vol of 3.0 ml of medium containing 1% fetal calf serum and 50 µg/ml ascorbate as described in Table I. After a 24-h incubation with 1 µCi/ml [^{14}C]proline, the secreted media proteins were analyzed for their total ^{14}C and [^{14}C]hydroxyproline content. The results are the average of duplicate determinations which agreed within 10% of one another.
‡ Values are [^{14}C]hydroxyproline per total ^{14}C × 100.
§ Final concentration of PHA in the fibroblast cultures was 0.13 µl/ml, the same concentration carried over into the fibroblast cultures in the stimulated mononuclear supernate.

Effects of Mononuclear Supernates on Collagen Synthesis and Cell Viability. To determine whether collagen synthesis was altered by mononuclear supernatants, fibroblast cultures were incubated with 1:7.5 dilutions of dialyzed supernatants from either PHA-stimulated or nonstimulated cultures. Table II demonstrates that the mononuclear cell preparations had little effect on total incorporation of [^{14}C]proline in the secreted proteins. However, there was a 90% inhibition of synthesis of [^{14}C]hydroxyproline, suggesting a decrease in the synthesis of collagen relative to other secreted proteins in the presence of the supernatant from the stimulated cells. The degree of hydroxylation decreased from 30% in the control cultures to 2.7% with the stimulated mononuclear supernate. The supernate from nonstimulated cells also caused a smaller but similar inhibition. At the concentrations employed, PHA by itself or added to supernatants from control mononuclear cells had no effect on the fibroblasts.

The fact that total incorporation was not affected suggested that the inhibition of [^{14}C]hydroxyproline synthesis was not a result of a general toxic effect on the cells, or a nonspecific inhibition of protein synthesis. This view was supported by studies on the viability of the cells using trypan blue exclusion as our test. At the end of the experiments described in Table II, the cells in replicate cultures which had been incubated in the presence or absence of the supernates were exposed to the dye (Table III). The viability of all the cultures was comparable, irrespective of whether they contained the supernates or not. In all cases, >90% of the cells were viable.

To assess the effects of varying concentrations of the mononuclear supernates, the fibroblast cultures were incubated with dilutions of the supernates ranging from 1:7.5 to 1:250. Again, total incorporation was not affected but the degree of hydroxylation of the proteins was progressively depressed by increasing concentrations of the mononuclear supernates (Fig. 1). A detectable effect was observed with a 1:60 dilution of the stimulated supernate and a 1:30 dilution of the nonstimulated supernate.

TABLE III

Effect of Mononuclear Supernate on Fibroblast Viability Measured by Trypan Blue Exclusion[*]

Mononuclear supernatant addition	Total cells	Viable cells	Viable
	× 10^{-3}	× 10^{-3}	%
None	332	314	94.5
PHA-stimulated	300	278	92.7
Non-stimulated	317	287	90.4
Non-stimulated plus PHA[‡]	306	298	97.4

[*] Confluent fibroblast cultures were incubated with 400 μl of the additions listed in the table in a total vol of 3.0 ml of medium containing 1% fetal calf serum as described in Table I and Materials and Methods. After a total incubation time of 72 h, cell viability was measured by trypan blue exclusion. The values represent the average of triplicate cultures.

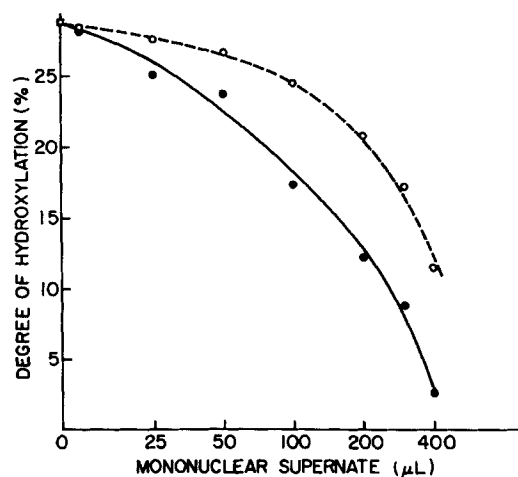[‡] Final concentration of PHA in the fibroblast cultures was 0.13 μl/ml.

FIG. 1.   Dependence of [^{14}C]hydroxyproline synthesis on concentration of mononuclear cell supernates. Confluent fibroblast cultures were incubated with the quantities of mononuclear cell supernates indicated in the figure in a total vol of 3.0 ml of medium containing 1% fetal calf serum and 50 μg/ml ascorbate as described in Table I and Materials and Methods. After a 24-h incubation with 0.5 μCi/ml [^{14}C]proline, the secreted proteins in the media were analyzed for their total ^{14}C and [^{14}C]hydroxyproline content. Total ^{14}C incorporation was unaffected by the supernates and was equal to 24,700 ± 2,600. The plotted values of the degree of hydroxylation represent the average of duplicate cultures which agreed within 10% of each other, e.g., 25.0 ± 2.5%. Cultures in the presence of stimulated supernate (●-●-●), nonstimulated supernate (O-O-O), or control cultures (□).

The results presented in this section are representative of those obtained with a majority of our supernatant preparations. With some supernates, qualitatively similar but not as large effects were found. In a few instances no inhibition of [^{14}C]hydroxyproline production was observed even at the highest concentration employed. However, in no case did we find stimulation of [^{14}C]hydroxyproline synthesis.

Collagenase Digestion of Labeled Proteins.   The effect on hydroxylation described above

TABLE IV

*Collagenase Digestion of Labeled Proteins**

Mononuclear supernatant addition	Total	Digested by collagenase	Digested
	$cpm \times 10^{-3}$	$cpm \times 10^{-3}$	%
None	68.5	39.8	58.1
Stimulated supernate	69.8	4.2	6.1
Non-stimulated supernate	71.8	20.4	28.4
Non-stimulated supernate plus PHA‡	67.0	17.8	26.6

* Confluent fibroblast cultures were incubated with 400 µl of each of the additions listed in the table in a total vol of 3.0 ml of medium containing 1% fetal calf serum and 50 µg/ml ascorbate for 48 h. The media were then replaced with fresh media containing the same additions but with 1 µCi/ml [^{14}C]proline and 100 µg/ml β-aminopropionitrile and the incubations continued for 24 h. The labeled secreted proteins were digested with highly purified bacterial collagenase (19), and the released peptides recovered as described in Materials and Methods. This collagenase preparation had no demonstrable proteolytic activity against noncollagenous protein.
‡ Final concentration of PHA in the fibroblast cultures was 0.13 µg/ml.

could be a result of either a decrease in the rate of collagen synthesis relative to other secreted proteins or a direct effect on hydroxylation. To differentiate the two possibilities, the labeled proteins from control cultures and those with mononuclear supernates were digested with highly purified bacterial collagenase to estimate total collagenous protein. The results in Table IV clearly show that the stimulated mononuclear supernate produced a dramatic decrease in total labeled collagenous protein. In the control, 58% of the label incorporated into secreted protein was digested by the collagenase. This figure dropped to 6% in the cultures with the stimulated supernate and 28% in the cultures with the nonstimulated supernate. These results demonstrate that the mononuclear cell supernates act by decreasing the relative rate of collagen synthesis rather than having a direct effect on proline hydroxylation. They also confirmed the observation that PHA added to cultures incubated in the presence of supernates from nonstimulated mononuclear cells had no effect on the synthesis of collagen by the fibroblasts.

Ascorbic Acid Determinations in Cultures. We previously have shown that collagen synthesis and accumulation by cultured dermal fibroblasts are strongly dependent on the presence of ascorbic acid in the incubation medium (22). To exclude the possibility that depletion of ascorbic acid by materials present in the mononuclear supernates was responsible for the observed effects, the amount of ascorbic acid remaining in the fibroblast culture media at the end of the labeling period was measured employing a microfluorometric assay (23). We found that the ascorbic acid content of media from fibroblasts cultured with or without mononuclear cell supernatants was not different and ranged between 10 and 14 µg/ml. The concentration at the beginning of the labeling period was 50 µg/ml. The levels of 10–14 µg/ml are adequate to sustain optimal collagen synthesis (24).

Chromatography of Labeled Proteins. The observation that total incorporation of [^{14}C]proline remained relatively constant whereas incorporation into collagenous polypeptides declined implied that incorporation into noncollagenous protein in-
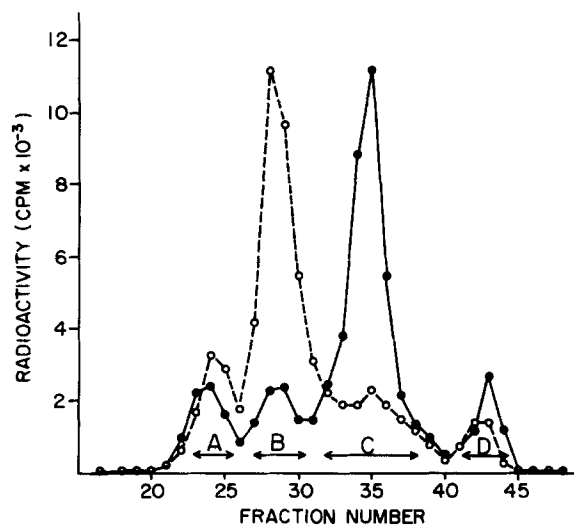
FIG. 2.   Gel filtration chromatography of labeled proteins. Fibroblast cultures were incubated under either control conditions or in the presence of a 1:7.5 dilution of a supernate prepared from PHA-stimulated mononuclear cells as described in Materials and Methods and Table II. The secreted labeled proteins were subjected to chromatography on a 1.5 × 85-cm column of 6% Agarose (Bio-Gel A-5m, 200–400 mesh). The fraction size was 2.0 ml. Marker β-component of rat tail collagen eluted at fraction 25, marker α-component of rat tail collagen at fraction 30 and bovine serum albumin at fraction 35. ●, +mononuclear supernate; ○, control.

creased. In an attempt to document this and to characterize the secreted proteins, they were chromatographed on columns of A-5-m in sodium dodecyl sulfate. The results obtained with the control cultures and those with stimulated mononuclear supernate are shown in Fig. 2. In the control chromatograph, a predominant peak containing ≅ 60% of the total radioactivity was found in the position of procollagen. The remaining 40% was found in three other peaks of varying molecular weight. In contrast, in the sample from the culture with the mononuclear supernate, 60% of the radioactivity was found in a peak of ≅ 68,000 mol wt, whereas the distinctly separate procollagen peak contained only ≅ 14% of the radioactivity in the chromatograph. Four regions of the chromatograph were pooled as indicated in Fig. 2 and these pooled samples were analyzed for their [14C]hydroxyproline content (Table V). In the control sample, >90% of the [14C]hydroxyproline was contained in the peak from the procollagen region. In the sample from the incubation with the stimulated supernate, 75% of the [14C]hydroxyproline was in the relatively minor procollagen peak. The balance of the [14C]hydroxyproline was found largely in peaks A and D and virtually no [14C]hydroxyproline was found in peak C. The distribution of radioactivity observed in these two chromatographs is consistent with the results reported above on the collagenase sensitivity of the secreted proteins and supports the concept that under these experimental conditions the major effect of the mononuclear supernates was to change the rate of collagen synthesis relative to that of other proteins.

Discussion

The studies reported here have shown that addition of supernates from cultured human mononuclear cells to monolayers of normal human dermal fibroblasts consist-

TABLE V

*Hydroxyproline Content of Proteins Secreted by Fibroblasts**

Fractions from agarose columns	Total ^{14}C	[^{14}C]Hyp	Degree of hydroxyl-ation‡
	cpm × 10^{-3}	*cpm × 10^{-3}*	%
Control			
A	8.7	0.2	2.2
B	37.0	14.5	39.2
C	12.1	0.5	4.5
D	4.0	0.4	8.8
Plus mononuclear supernate			
A	7.5	0.2	2.6
B	8.0	2.7	34.3
C	35.6	0.3	0.8
D	8.8	0.4	4.4

* Confluent fibroblast cultures were incubated with 2 μCi/ml [^{14}C]proline with or without 0.3 ml supernate from PHA-stimulated mononuclear cells in 3.0 ml of medium for 24 h as described in Materials and Methods and Table I. Samples of the secreted proteins were chromatographed on Agarose A-5m and the peaks which were obtained were pooled as designated in Fig. 2. After hydrolysis, the proteins were analyzed for their [^{14}C]hydroxy-proline content (20).

‡ Values are [^{14}C]hydroxyproline per total ^{14}C × 100.

ently resulted in significant and selective inhibition of collagen synthesis by these cells. Similar effects were demonstrated with supernates from either nonstimulated or PHA-stimulated mononuclear cells but significantly greater inhibition was obtained with the supernates of lectin-stimulated cells. (Table II). As little as a 1:60 dilution of the stimulated supernate resulted in a significant decrease in [^{14}C]hydroxyproline synthesis. These results differ from previous reports of other laboratories which showed increased collagen synthesis by fibroblasts incubated in the presence of extracts obtained from silica-stimulated peritoneal macrophages (8), lymphokines obtained from PHA-stimulated human peripheral blood lymphocytes (13) or supernates from antigen-stimulated guinea pig lymphocytes (14).

Although the methods employed by these authors were similar to the methods we used for our experiments, in most cases there were either differences in the species of cells used or differences in the culture conditions of the fibroblast target cells which may partially explain the variation in results. Perhaps most significantly, we incubated the fibroblasts in media containing 1% fetal calf serum throughout the duration of the incubation with the mononuclear supernatants whereas in some of the experiments of other workers the fibroblasts were incubated in the absence of serum. It has been our experience that fibroblasts in culture demonstrate a selective requirement for serum for optimal collagen synthesis which is manifested even in confluent cultures and therefore appears to be independent of serum effects on cell proliferation (25). It can be seen that marked stimulation of total [^{14}C]proline incorporation and [^{14}C]-hydroxyproline synthesis occurred when the confluent cultures were supplemented with serum (Table I). Other authors have also reported similar results of serum supplementation with fibroblasts or smooth muscle cells (26–29). It is possible, therefore, that cells incubated under conditions of serum deprivation may vary in

their responses to stimuli compared to fibroblasts in serum-supplemented medium. We have found in experiments not reported here that collagen synthesis by fibroblasts incubated in the absence of serum could be stimulated by addition of mononuclear cell supernates. Our results are similar to those obtained by Harrington et al. (10) who found that supernates and extracts of silica-exposed hamster macrophages markedly reduced the amount of collagen associated with the cell layer in fibroblasts cultured in the presence of serum.

To exclude the possibility that the decreased [^{14}C]hydroxyproline synthesis observed here was a result of cytotoxic effects of the supernates on the fibroblasts, we examined the viability of the fibroblasts after 3 d of incubation with the supernates. Our results failed to show any significant cytotoxic effect and >90% of the cells remained viable as determined by trypan blue exclusion under the conditions of our experiments (Table III). Wahl et al. (14) also reported no cytotoxic effects of their mononuclear cell supernates on fibroblasts, although Johnson and Ziff (13) found by qualitative observation that supernates from stimulated mononuclear cells decreased the number of cells in their fibroblast cultures. It could also be argued that the decrease in [^{14}C]-hydroxyproline synthesis demonstrated in the present study did not represent a decrease in the amount of collagen synthesized but simply underhydroxylation of the collagen molecules. This possibility was eliminated since the amount of collagenase-sensitive macromolecules was markedly reduced in the experimental cultures when compared to controls (Table IV).

One remarkable finding of our studies was the demonstration that the mononuclear cell supernatants caused a change in the pattern of macromolecules synthesized by the fibroblast monolayers. As shown in the chromatograms presented in Fig. 2, in the control fibroblast cultures most of the incorporated [^{14}C]proline eluted in the position of procollagen (Peak B), whereas, in cultures incubated in the presence of mononuclear cell supernates, there was a marked reduction in the amount of procollagen which was accompanied by a concomitant increase in a proline-labeled species (Peak C) with an apparent mol wt of ≅68,000. This protein(s) was noncollagenous in nature as indicated by the absence of [^{14}C]hydroxyproline (Table V) and its resistance to digestion with bacterial collagenase. The nature and significance of this macromolecule are not known at present but it is possible that clarification of its role may be of relevance to a further understanding of the mechanisms involved in the interaction of inflammatory cells with fibroblasts in the development of a fibrotic response. In the experiments of Wahl, et al. (14), supernates from antigen-stimulated lymphocytes increased fibroblast synthesis of both collagen and noncollagenous protein but the stimulation of synthesis of noncollagenous protein was greater. Supernates from nonstimulated lymphocytes appeared to actually decrease collagen synthesis by 50% compared to untreated fibroblast cultures.

Based on our results and those of others, we suggest that during the development of an inflammatory response, the participation of the connective tissue cells occurs in two phases. Initially, there is stimulation of fibroblast proliferation perhaps mediated through factors released by platelets and other cells. Accumulation of collagen and other biosynthetic products would then occur. Subsequently when the fibroblastic response is adequate and repair is complete, inhibitory factors such as the one described here would terminate the deposition of collagen. Normally, there is an

adequate balance between stimulation and inhibition which is strictly maintained but which may be perturbed under certain conditions resulting in either deficient or excessive accumulation of connective tissue macromolecules.

Summary

Mononuclear cell infiltration and alteration in the connective tissues are prominent features of the inflammatory response in a number of diseases. To determine whether mononuclear cell products can modulate collagen synthesis, human peripheral mononuclear cells from normal donors were isolated by Ficoll-Hypaque gradient centrifugation and then incubated for 48 h with or without phytohemagglutinin. Confluent cultures of normal, human skin fibroblasts were incubated with [^{14}C]proline and various amounts of dialyzed supernates from the mononuclear cell cultures. Labeled, newly synthesized collagen was estimated by [^{14}C]hydroxyproline analysis, collagenase digestion, and chromatography on Agarose A-5m in sodium dodecyl sulfate. The total incorporation of [^{14}C]proline was not significantly affected by addition of the mononuclear cell supernates, but as much as a 90% decrease in the synthesis by the fibroblasts of labeled collagen was found relative to controls. Supernates from the phytohemagglutinin-stimulated cultures were more active than those from nonstimulated cells. These results suggest that mononuclear cells can synthesize a factor(s) which can selectively inhibit collagen synthesis.

References

1. Arey, L. B. 1936. Wound healing. *Physiol. Rev.* **16:**327.
2. Todaro, G. J., Y. Matusuya, S. Bloom, A. Robbins, and H. Green. 1967. *In* Growth Regulating Substances for Animal Cells in Culture. Defendi, V. and M. Stocker, editors. The Wistar Institute Press, Philadelphia, Pa. 87.
3. Wiebel, F., and R. Baserga. 1969. Early alterations in amino acid pools and protein synthesis of diploid fibroblasts stimulated to synthesize DNA by addition of serum. *J. Cell. Physiol.* **74:**191.
4. Holley, R. W., and J. A. Kiernan. 1974. Control of the initiation of DNA synthesis in 3T3 cells: Serum factors. *Proc. Natl. Acad. Sci. U. S. A.* **71:**2980.
5. Chen, L. B. and J. M. Buchanan. 1975. Mitogenic activity of blood components. I. Thrombin and prothrombin. *Proc. Natl. Acad. Sci. U.S.A.* **72:**131.
6. Gospodarowicz, D. and J. S. Moran. 1976. Growth factors in mammalian cell culture. *Ann. Rev. Biochem.* **45:**531.
7. Carrel, A. 1922. Growth-promoting function of leucocytes. *J. Exp. Med.* **35:**385.
8. Heppleston, A. G. and J. A. Styles. 1967. Activity of a macrophage factor in collagen formation by silica. *Nature (Lond.).* **214:**521.
9. Yaron, M. and C. W. Castor. 1969. Leukocyte-connective tissue cell interaction. I. Stimulation of hyaluronate synthesis by live and dead leukocytes. *Arthritis Rheum.* **12:** 365.
10. Harrington J. S., M. Ritchie, P. O. King, and K. Miller (1973) The in-vitro effects of silica-treated hamster macrophages on collagen production by hamster fibroblasts. *J. Pathol.* **109:** 21.
11. Aalto, M., and E. Kulonen. 1974. Inhibition of protein synthesis in tendon cells by extracts from experimental granulation tissue. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **49:**70.

12. Aalto, M., M. Potila, and E. Kulonen. 1976. The effect of silica-treated macrophages on the synthesis of collagen and other proteins in vitro. *Exp. Cell Res.* **97**:193.
13. Johnson, R. L., and M. Ziff. 1976. Lymphokine stimulation of collagen accumulation. *J. Clin. Invest.* **58**:240.
14. Wahl, S. M., L. M. Wahl, and J. B. McCarthy. 1978. Lymphocyte-mediated activation of fibroblast proliferation and collagen production. *J. Immunol.* **121**:942.
15. Ross, R. 1968. The fibroblast and wound repair. *Biol. Rev. Camb. Philos. Soc.* **43**:5196.
16. Boyum, A. 1968. Separation of leucocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):31.
17. Oppenheim, J. J., S. Dougherty, S. C. Chan, and J. Baker. 1975. *In* Laboratory Diagnosis of Immunological Disorders. G. N. Vyas, editor. Grune & Stratton, New York. 87.
18. Bashey, R. I., S. A. Jimenez, and J. S. Perlish. 1977. Characterization of secreted collagen from normal and scleroderma fibroblasts in culture. *J. Mol. Med.* **2**:153.
19. Miyoshi, M., and J. Rosenbloom. 1974. General proteolytic activity of highly purified preparations of clostridial collagenase. *Connect Tissue Res.* **2**:77.
20. Juva, K., and D. J. Prockop. 1966. Modified procedure for the assays of H^3-or C^{14}-labeled hydroxyproline. *Anal. Biochem.* **15**:77.
21. Jimenez, S. A., P. Dehm, and D. J. Prockop. 1971. Further evidence for a transport form of collagen. Its extrusion and extracellular conversion to tropocollagen in embryonic tendon. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **17**:245.
22. Jimenez, S. A., and R. I. Bashey. 1977. Collagen synthesis by scleroderma fibroblasts in culture. *Arthritis Rheum.* **20**:902.
23. Feng, J., A. H. Melcher, D. M. Brunette, and H. K. Moe. 1977. Determination of L-ascorbic acid levels in culture medium; concentrations in commercial media and maintenance of levels under conditions of organ culture. *In Vitro.* **13**:91.
24. Blanck, T. J. J., and B. Peterkovsky. 1975. Stimulation of collagen secretion by ascorbate as a result of increased proline hydroxylation in chick embryo fibroblasts. *Arch. Biochem. Biophys.* **171**:259.
25. Bashey, R. I., and S. A. Jimenez. 1977. Increased sensitivity of scleroderma fibroblasts in culture to stimulation of protein and collagen synthesis by serum. *Biochem. Biophys. Res. Commun.* **76**:1214.
26. Bankowski, E., and N. M. Mitchell. 1973. Human procollagen. I. An anionic tropocollagen precursor from skin fibroblasts in culture. *Biophys. Chem.* **1**:73.
27. Narayanan, A. S., and R. C. Page. 1977. Serum modulates collagen types in human gingiva fibroblasts. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **80**:221.
28. Burke, J. M., and R. Ross. 1977. Collagen synthesis by monkey arterial smooth muscle cells during proliferation and quiescence in culture. *Exp. Cell Res.* **107**:387.
29. Nolan, J. C., G. J. Cardinale, and S. Udenfriend. 1978. The formation of hydroxyproline in collagen by cells grown in the absence of serum. *Biochim. Biophys. Acta.* **543**:116.