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Synthesis of Type I and Type III Collagen by Synovial Cells in Tissue Culture Derived from Patients with Rheumatoid Arthritis, Osteoarthritis, and Normal Individuals (40982)[1]

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Abstract. Synovial tissues obtained from patients with rheumatoid arthritis, osteoarthritis, or traumatic damage (normal) were placed in culture, using the explant technique. The resulting monolayer cultures of cells were found to synthesize Type I and Type III collagen. In rheumatoid derived tissues labeled in primary organ culture, 31% of the mean total collagen produced was Type III. Rheumatoid synovial cells growing in monolayer culture at the second passage synthesized 18 ± 2% Type III collagen compared to 15 ± 2% in respective control cultures. The mean percentage of Type III collagen synthesized by rheumatoid synovial cells at the fourth passage in culture was 20 ± 3% compared to 14 ± 2% in fourth passage cells derived from osteoarthritic tissue or 13 ± 3% in fourth passage cells derived from normal human synovium. These data indicate that an increase commitment of rheumatoid synovium for Type III collagen synthesis, expressed in primary organ cultures, is not detectable in early passage cells growing in monolayer. Therefore, factors in the local tissue environment may participate in the modulation of collagen heterogeneity in the rheumatoid synovium.

The sequence of events leading to joint destruction in patients with rheumatoid arthritis has been investigated extensively. The chronic inflammation within the joints is associated with a proliferation of the synovial membrane cells leading to the formation of invasive pannus tissue. This tissue has the capacity to release proteolytic enzymes, such as collagenase, that erode the cartilage and contribute to joint destruction (1).

Collagen is the major connective tissue protein and as such plays an important structural role in the organization of the extracellular matrix of the synovial membrane and pannus. Collagen provides sites for cell attachment (2) and may influence the migration (3, 4) and proliferation of fibroblasts (5). Various types of collagen have been characterized: Type I, Type II, Type III, and Type IV are the most abundant (6). More recently other collagen types that occur in relatively small amounts have been reported; these have been designated as either α[A], α[B], and α[C] chains (7, 8) or X and Y chains (9). A high proportion of Type III collagen has been demonstrated in rat dermal granulation tissue compared to normal skin (10).

Data implicating changes in collagen heterogeneity as a chemical lesion in rheumatoid synovium are conflicting at present. Weiss *et al.* (11) reported finding a high ratio of Type III to Type I collagen in inflamed and rheumatoid synovial tissue compared to normal synovial tissue. They recovered abnormal amounts of Type III collagen from inflamed and rheumatoid synovial tissue as an acetic acid extractable polymeric form. Of the remaining collagen within the synovial tissues, 10–15% was pepsin extractable and most of this was also Type III collagen. These authors suggested that the amount of Type III collagen might

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be related to the degree of inflammation. However, Eyre and Muir (12) using a cyanogen bromide extraction procedure which extracted a much greater percentage of the total collagen within the synovial tissue, found that the proportion of Type III to Type I collagen was similar in both normal and rheumatoid synovial tissue. They did agree that Type III collagen in rheumatoid synovial tissue is more susceptable to pepsin extraction, perhaps signifying a lesser degree of crosslinking and an increased rate of metabolism. Brown *et al.* (8) have recently extracted collagen α[A], α[B], and α[C] chains from synovial tissue. These collagen types were relatively minor components of the total pepsin extractable collagen, but they could be present in greater amounts as unextractable collagen. In these studies the relative amounts of these pepsin extractable components remained unchanged in normal and rheumatoid synovial tissue (8).

The project reported here was conducted to examine the relative rates of synthesis of collagen Types I and III by synovial cells growing in culture, using cells derived from patients with rheumatoid arthritis, osteoarthritis, or from normal individuals undergoing surgery as the result of recent trauma.

Methods and materials. Patients and synovial tissue. Synovial tissue was obtained from the joints of patients during reconstructive and restorative surgery at the Roger Williams General Hospital, Providence, Rhode Island. Normal (nonrheumatoid) tissue was obtained from patients that had simple joint damage, usually a torn meniscus. Synovial tissue identified as rheumatoid was obtained from patients fulfilling the American Rheumatism Association's criteria for rheumatoid arthritis. The rheumatoid patients all had advanced erosive stages of inflammatory polyarthritis that characterizes the disease and which resulted in significant functional impairments in each case. In each instance the impairment required surgical intervention and in all cases histological examination of the excised tissue revealed chronic synovitis consistent with rheumatoid arthritis. All the patients with osteoarthritis had a progressive course of noninflammatory arthritis involving a weight-bearing joint. In each case the patient required surgery because of significant disability with a radiological assessment of moderate to severe osteoarthritis of the involved hip or knee according to the classification of Kellgren and Lawrence (13). The tissues obtained from the patients with osteoarthritis were also examined histologically and found to be consistent with the diagnosis.

One patient from the normal group (NS 3), one patient with osteoarthritis (OA 3), and two patients with rheumatoid arthritis (RA 2 and RA 6) were on nonsteroidal anti-inflammatory drugs at the time of surgery. One patient with rheumatoid arthritis (RA 4) was receiving maintenance gold and 10 mg prednisone daily at the time of surgery. In each of the other cases the patient had not been receiving anti-inflammatory medication within 3 months of the surgical procedure when the synovial tissue was obtained for study.

Culture of cells. The tissue obtained during surgery was dissected free of any excess fat, capsular, and cartilage tissue, and finely cut into approximately 1-mm^3 pieces; about 15 tissue pieces were placed in each 100 × 20-mm tissue culture dish. The cells were grown in Dulbecco's modified Eagles (DME) media containing 10% (v/v) heat-denatured fetal bovine serum (FBS), penicillin–streptomycin solution (100 units penicillin and 100 μg streptomycin/ml media) and Fungizone (2.5 μg/ml media) (all obtained from Grand Island Biological Co., Grand Island, N.Y.) as previously described (14). After 1 or 2 weeks the cells could be seen growing outward from the tissue pieces and after approximately 4 weeks the cells became confluent and were subcultured into 75-cm^2 tissue culture flasks. To subculture (passage) the cells, the media and original tissue fragments were removed by suction and the cell layer detached from the dish by incubation for 5 min in isotonic phosphate-buffered saline (PBS) (pH 7.0) containing 0.1% typsin (Grand Island Biological Co.), 0.2 mg/ml glucose, and 0.2 mg/ml ethylenediaminetetraacetic acid (EDTA). After detachment, the cells were resuspended in

fresh media and replated. Two weeks after the first passage the synovial cells were subcultured again and this time replated at one-third of their confluent density. The cells were usually confluent again and subcultured after a further 2-week period. The cell medium was normally replaced with fresh media every 4 days. All experiments were performed with cells at either one, two or four passages. The cell cultures were tested for and found to be free of mycoplasma contamination using a fluorescent assay (Bioassay Systems, Cambridge, Mass.).

Collagen biosynthesis and extraction. Cultures of confluent synovial cells in 75-cm^2 flasks were incubated for 4 days in 10 ml DME containing 10% (v/v) FBS, antibiotics, and Fungizone. L[^{14}C]Proline (10 μCi; sp act, 260/mmole; Schwarz/Mann, Orangeburg, N.Y.), 1 mg of 3-aminopropionitrile fumarate (BAPN) (to prevent collagen crosslinking), and 10^{-4} *M* sodium ascorbate (to insure complete hydroxylation and secretion) were added to the culture media. Freshly prepared sodium ascorbate (10^{-4} *M*) was added daily to the culture. At the end of the incubation period the media were removed and the collagen precipitated by the addition of ammonium sulfate to 50% saturation. In the presence of ascorbate and BAPN, the cell layer was found to contain less than 5% of the radioactive hydroxyproline. After centrifugation the precipitate was redissolved in 3 ml of 7% (v/v) acetic acid and dialyzed against 7% (v/v) acetic acid. The solution containing collagen was then incubated for 3 hr at room temperature with pepsin (100 μg/ml; Worthington, Freehold, N.J.). For analysis of collagen heterogeneity by polyacrylamide gel electrophoresis, the collagen samples were dialyzed at 4° against 0.01 *M* sodium phosphate buffer (pH 7.2). For analysis of collagen heterogeneity by carboxymethyl cellulose chromatography, the collagen samples were dialyzed at 4° against 0.06 *M* sodium acetate buffer (pH 4.8).

Primary cultures of rheumatoid tissue obtained directly from surgery were cut into 1-mm^3 pieces of tissues as described above. Approximately 20 tissue pieces were incubated for 24 hr in 2 ml DME media containing antibiotics. L[^{14}C]Proline (10 μCi; sp act; 260 mCi/mmole), 200 μg BAPN, and 10^{-4} *M* sodium ascorbate were added to the medium. At the end of the incubation period the medium and tissue fragments were homogenized and the collagen extracted, as described above, by precipitation in ammonium sulfate and incubation with pepsin. Finally, the collagen samples were dialyzed against 0.01 *M* sodium phosphate buffer (pH 7.2) and then analyzed by polyacrylamide gel electrophoresis.

The type of collagen present in the synovial tissue obtained from surgery was determined by sequential extraction of the tissue at 4° overnight with (i) 0.5 *M* acetic acid; (ii) 0.5 *M* acetic acid containing 100 μg/ml pepsin; (iii) 0.5 *M* acetic acid containing 10 mg/ml pepsin. After the extraction the pepsin was inactivated by raising the pH of the extract to 7.4 with sodium hydroxide. The collagen was then purified by repeated precipitation with 0.9 *M* sodium chloride in 0.5 *M* acetic acid. The precipitated collagen was dialyzed against 0.01 *M* sodium phosphate buffer (pH 7.2) and analyzed by polyacrylamide gel electrophoresis as described below. The Coomassie brilliant blue-stained bands of the gel corresponding to standard collagen α chains were estimated using a recording Gilford gel scanner (Model 2520) and a Numonics electronic planimeter. The peak areas derived were compared to those of standard purified collagen α chains to estimate the amount of collagen in the extract. The collagen standards used were purified from bovine calf skin as previously described (15).

The total amount of collagen present in osteoarthritic and rheumatoid synovial tissue was estimated by analysis of hydroxyproline content as described by Kivirikko *al.* (16).

Analysis of collagen types. (*a*) *Polyacrylamide gel electrophoresis.* The sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) procedure of Furthmayr and Timpl (17) was used for analysis of collagen extracted from synovial tissue and ^{14}C-labeled collagen synthesized by the synovial tissue pieces *in vitro*. The

SDS–PAGE procedure of Neville (18) was used for analysis of ^{14}C-labeled collagen synthesized by synovial cells in culture, since this procedure was later found to be an improvement on the Furthmayr and Timpl (17) procedure. Standards of Type I collagen (10 μg) and Type III collagen (10 μg) were added to each ^{14}C-labeled collagen sample before electrophoresis. This enabled visualization of the collagen bands within the gel, after electrophoresis, by staining with Coomassie brillant blue R-250 (Eastman Kodak, Rochester, N.Y.). The Type I and Type III collagen standards were prepared from pepsin extracted calf skin collagen in our laboratory as previously described (15). It was established that the calf skin collagen components had electrophoretic mobilities similar to human collagen. The ^{14}C-labeled collagen samples obtained from incubation of the synovial tissue pieces already contained ample collagen for visualization by staining with Coomassie brilliant blue and addition of calf skin collagen was not required in this case.

The percentage of Type I and Type III collagen was calculated as previously described (19). The determination of the ratio of labeled Type I to Type III collagen was based on the observation that Type I collagen is a trimer of two α1 (I) chains and one α2 chain, while Type III collagen is a trimer of three α1 (III) chains (6). The α1 (I) and α2 chains migrate at different rates (14). Under nonreducing conditions the α1 (III) chains migrate as high-molecular-weight trimers, since the chains are held together by disulfide bonds, but under reducing conditions (1% (v/v) mercaptoethanol per sample) the α1 (III) chains migrate as separate entities at a rate similar to that of α1 (I) chains (20). Therefore, each ^{14}C-labeled collagen sample analyzed was electrophoresed under both nonreducing and reducing conditions. After staining the collagen bands (of the coelectrophoresed collagen standard) with Coomassie brilliant blue, the bands staining for α1 and α2 chains were sliced from the gel and dissolved by heating at 60° in the presence of 0.2 ml of hydrogen peroxide (30% (v/v)). Hydromix (Yorktown Research, N.J.) was added to each sample and the radioactivity counted in a Packard liquid scintillation counter.

(*b*) *Carboxymethyl cellulose chromatography.* ^{14}C-Labeled collagen samples were analyzed by carboxymethyl cellulose chromatography using the procedure described by Miller (21). Briefly, a 1.8 × 10-cm column (Pharmacia, Uppsala, Sweden) was filled with carboxymethyl cellulose (Whatman, Kent, England) equilibrated in 0.06 *M* sodium acetate buffer (pH 4.8) and maintained at 42°. The ^{14}C-labeled collagen samples containing 2–4 mg of Type I and Type III purified calf skin collagen as carrier were denatured by heating at 56° for 30 min in the presence of 10 *M* urea. The samples were reduced by the addition of 1% (v/v) mercaptoethanol and applied to the column. The collagen components were eluted from the column using a linear salt gradient; starting buffer consisted of 200 ml 0.06 *M* sodium acetate buffer (pH 4.8); final buffer consisted of 200 ml starting buffer containing 0.2 *M* sodium chloride. The elutant was collected in 12 ml fractions; 1 ml of each fraction was mixed with 10 ml Hydromix and the radioactivity counted. The percentage Type I and percentage Type III ^{14}C-labeled collagen were calculated from the radioactivity eluted in the peaks that corresponded to α1 (I), α1 (III), and α2 chains (Fig. 1). The α1 (I), α1 (III), and α2 peaks were confirmed by their migration as α1 or α2 bands on SDS–PAGE.

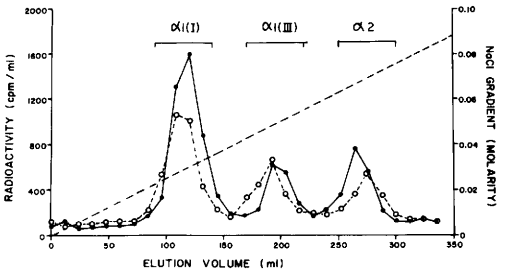

FIG. 1. A typical carboxymethyl cellulose chromatography elution profile of acetic acid and pepsin (100 μg/ml) extracted ^{14}C-labeled collagen. ●——●, collagen synthesized by cells cultured from patient NS 2 (passage 1); ○– – –○, collagen synthesized by cells cultured from patient RA5 (passage 1) (see Table II). The diagonal dashed line represents the linear sodium chloride gradient.

Results. The amount of collagen in rheumatoid and osteoarthritic (non-rheumatoid control) tissue varied from 20 to 45% of the dry weight of the tissue, as estimated by hydroxyproline analysis. Using acetic acid and the acetic acid with pepsin (100 μg/ml), it was possible to extract 9% of the total collagen from rheumatoid synovial tissue and 6% of the total collagen from osteoarthritic synovial tissue (Table I). Using acetic acid with pepsin (10 mg/ml), it was possible to extract a further 81% of the total collagen from rheumatoid synovial tissue, but only a further 5% total collagen from osteoarthritic synovial tissue (Table I). The pooled extractable collagen components isolated from the rheumatoid synovial tissue were estimated to be 23% Type III collagen after separation by SDS–PAGE, whereas similar analysis of collagen from osteoarthritic synovial tissue revealed 18% Type III collagen (Table I). The typical SDS–PAGE patterns of the collagen extracted from rheumatoid synovial tissue using acetic acid with pepsin (10 mg/ml) are shown in Fig. 2. An additional band, possibly α[B] or α[C] collagen chains, can be seen between the β dimers and α1 chain position (Fig. 2). Incubation of the collagen extracts with purified bacterial collagenase before electrophoresis removed all traces of the bands which were stained for protein (Fig. 2).

Normal synovial cells in culture at the first or second passage synthesized 13–24% (mean 19%) Type III collagen when the radioactive α chains were separated by carboxymethyl cellulose (CMC) chromatography, and 10–17% (mean 15%) Type III collagen when separated by SDS–PAGE. These means do not include 42 and 38% Type III collagen (analyzed by CMC chromatography and SDS–PAGE, respectively) synthesized by normal synovial cells (at first passage) from a 9-year-old patient (Table II, Fig. 3). The amount of Type III collagen synthesized by osteoarthritic synovial cells in culture at the first

TABLE I. RELATIVE PERCENTAGE OF TOTAL COLLAGEN AND COLLAGEN TYPES FROM ACETIC ACID AND PEPSIN EXTRACTS OF RHEUMATOID ARTHRITIC AND OSTEOARTHRITIC SYNOVIAL TISSUES.

	Extraction procedure								
	Acetic acid (0.5 *M*)			Acetic acid (0.5 *M*) + pepsin (100 μg/ml)			Acetic acid (0.5 *M*) + pepsin (10 mg/ml)		
% Total collagen extracted from RA tissues	4.0			5.3			80.7		
% Total collagen extracted from OA tissues	2.4			4.0			5.2		
	Collagen type			Collagen type			Collagen type		
	I	III	Other	I	III	Other	I	III	Other
% Collagen type in RA tissue extract	89	10	0	61	38	0	58	23	20
% Collagen type in OA tissue extract	58	2	39	51	25	25	47	20	34
Collagen type in RA extract as % total extracted	3.9	0.4	0	3.6	2.2	0	52.2	20.7	18.0
Collagen type in OA extract as % total extracted	12.2	0.4	8.2	17.3	8.5	8.5	21.2	9.0	15.3

Note. RA and OA represent synovial tissue from rheumatoid arthritic and osteoarthritic patients, respectively. Rheumatoid arthritic synovial tissue was pooled in equivalent amounts from three patients and osteoarthritic synovial tissue, from two patients. Estimation of percentage total extracted was determined by the hydroxyproline content solubilized relative to the hydroxyproline content of whole tissue. Collagen types were determined by densiometric scans of collagen α chains before and after reduction in SDS–PAGE. Values are the means of duplicate analysis.

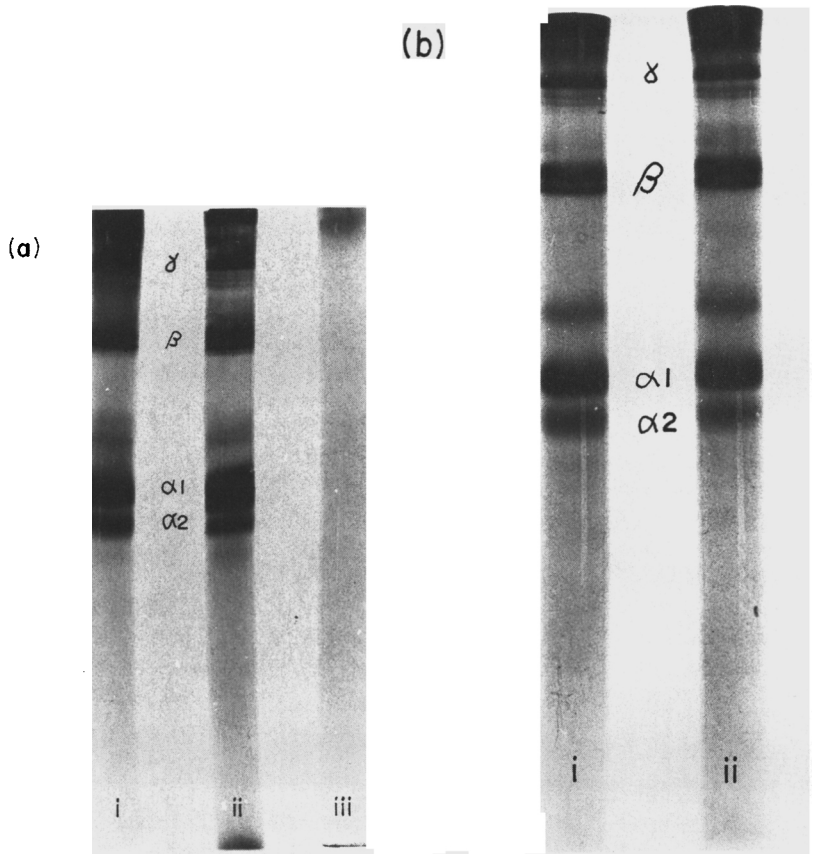

FIG. 2. SDS–polyacrylamide gel electrophoresis patterns of collagen (stained with Coomassie blue) extracted from rheumatoid synovial tissue by (a) acetic acid (0.5 *M*) with pepsin (100 μg/ml) followed by (b) acetic acid (0.5 *M*) with pepsin 10 mg/ml). (i) Sample unreduced (ii) sample reduced with 1% (v/v) mercaptoethanol (iii) sample (unreduced) incubated with excess purified bacterial collagenase for 3 hr.

passage ranged from 14 to 26% (mean 19%) by CMC chromatography and 8 to 23% (mean 15%) by SDS–PAGE (Table II, Fig. 3). Rheumatoid synovial cells in culture at the first or second passage synthesized slightly more Type III collagen, that is 15–34% (mean 25%) by CMC chromatography analysis and 8–29% (mean 18%) by SDS–PAGE (Table II, Fig. 3). The subsequent subculture of the rheumatoid synovial cells up to the fourth passage appeared to lead to a small loss in the percentage of Type III collagen, whereas subculture of the rheumatoid synovial cells up to the fourth passage, in most cases, appeared to enhance the percentage of Type III collagen. However, these progressive changes with passage number were not found to differ statistically from the first passage. The percentage of Type III collagen synthesized by rheumatoid synovial cells at the fourth passage was significantly higher ($P < 0.05$) than the percentage of Type III collagen synthesized by normal synovial cells at the fourth passage (Table II, Fig. 3). No changes were seen in the percentage of Type III collagen synthesized by osteoarthritic cells between the first and fourth passage (Table II, Fig. 3).

Analysis of Type III collagen by CMC chromatography resulted in a consistently higher value (up to 10% higher) than analysis by SDS–PAGE (Table II). In the case of SDS–PAGE analysis, this discrep-

TABLE II. COLLAGEN SYNTHESIZED BY SYNOVIAL CELLS GROWN FROM EXPLANTS OF SYNOVIAL TISSUE.

Patient No.	Age and sex	Biopsy site	Passage No.	Percentage type: CMC separation I	CMC separation III	CMC separation Other	SDS–PAGE separation I	SDS–PAGE separation III	SDS–PAGE separation Other
NS1	42 M	Great toe	1	58	13	30	83	17	0
			4				87	13	0
NS2	59 F	Knee	1	73	20	8	83	16	1
			4				91	10	0
NS3	19 F	Knee	1	75	24	0	76	17	7
			4				83	16	2
NS5	38 F	Thumb	1		N.D.		89	10	1
			4					N.D.	
NS mean ± SEM	40 ± 8		1 or 2	69 ± 5	19 ± 3	13 ± 9	83 ± 3	15 ± 2	2 ± 2
			4				87 ± 2	13 ± 3	1 ± 1
OA1	65 F	Hip	1	62	16	22	77	14	9
			4					N.D.[a]	
OA2	67 F	Hip	1	66	14	19	77	8	15
			4	67	14	19	78	9	13
OA3	73 F	Knee	1	53	26	20	66	23	11
			4				78	12	11
OA4	69 F	Hip	1		N.D.		76	10	14
			4				76	16	8
OA5	60 F	Knee	1		N.D.		72	18	10
			4				76	18	6
OA mean ± SEM	67 ± 2		1	60 ± 4	19 ± 4	20 ± 1	74 ± 2	15 ± 3	12 ± 1**
			4				77 ± 1	14 ± 2	10 ± 2**
RA1[a]	60 F	Right hip	2	67	14	8	90	8	
			4				82	18	0
RA2	53 F	Knee	2		N.D.		86	13	2
			4				77	16	7
RA3	69 M	Hip	1	76	24	0	75	17	8
			4				75	21	4
RA4	26 F	Wrist	1	52	34	14	69	29	4
			4				68	32	4
RA5[b]	60 F	Left hip	1	63	28	19	80	19	1
			4				65	27	8
RA6	60 F	Knee	1		N.D.		75	17	8
			4				87	8	6
RA7[b]	61 F	Right hip	2		N.D.		78	21	2
			4					N.D.	
RA mean + SEM	56 ± 5		1 or 2	64 ± 5	25 ± 4	10 ± 4	79 ± 3	18 ± 2	4 ± 2
			4				76 ± 3	20 ± 3*	5 ± 1**

Note. NS, OA, and RA represent synovial cell cultures obtained from "normal," osteoarthritic, and rheumatoid arthritic synovial tissue. Values for CMC are from pooled medium of two 75-cm^2 flasks with one or two determinations for each. Values for SDS–PAGE are from duplicate or triplicate determinations of one or two 75-cm^2 flasks each.

[a] Data not determined.

[b] Cells obtained from the same patient on different dates.

* $P < 0.05$, ** $P < 0.01$ when compared to equivalent NS group using Student's t test.

ancy may be due to the reduction of some disulfide bonds within Type III collagen during separation and prior to analysis which would decrease the estimation of Type III collagen by this technique. In the CMC chromatography separation, some of the collagen seen eluting in the Type III position may be α[A], [B], or [C] chains (8) which would increase the amount of Type III collagen estimated by this technique. However, linear regression analysis of data generated from all samples by these two

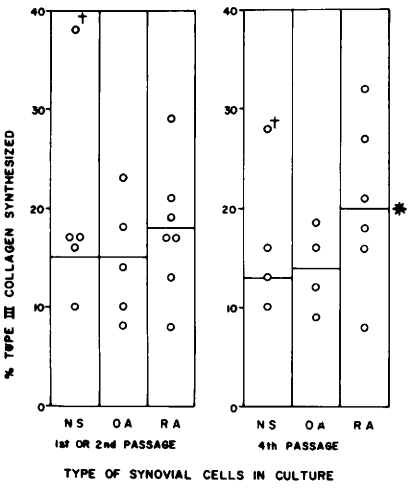

FIG. 3. The percentage of Type III collagen synthesized by synovial cells in culture was analyzed by SDS–polyacrylamide gel electrophoresis of acetic acid and pepsin extracted ^{14}C-labeled collagen. NS, OA, and RA synovial cells represent confluent cultures of cells grown from explants of synovium obtained from normal, osteoarthritic, and rheumatoid arthritic patients, respectively. Each point represents the result from a culture grown from an individual joint biopsy. Horizontal bars represent the mean values for each group. * signifies a significant difference ($P < 0.05$) between the RA and NS cultures. † represents cultures grown from the synovium of a 9-year-old patient; these results were not included in calculated mean values, because of the considerable age difference of this patient.

methods revealed a correlation coefficient of $r = 0.93$ indicating that the two separation methods are in agreement in detecting relative differences in collagen heterogeneity.

Rheumatoid synovial tissue fragments (from four patients) when incubated with labeled proline and BAPN in primary culture synthesized 31 ± 5% Type III collagen as separated and analyzed by SDS–PAGE. This was significantly higher ($P < 0.05$) than the amount of Type III collagen (18%) synthesized by rheumatoid synovial cells in culture at the first passage. When this procedure was employed on tissues derived from nonrheumatoid patients the limited amount of tissue, and the poor (compared to monolayer cultures) efficiency of this system in incorporating label, precluded the accurate estimation of the proportion of Type III collagen synthesized. It was not possible to obtain enough labeled collagens from this organ culture for CMC chromatography analysis for similar reasons.

Analysis of both SDS–PAGE and CMC chromatography of presumably Type I collagen synthesis gave ratios $\alpha 1$ (I)/$\alpha 2 > 2$. The excess of $\alpha 1$ (I) collagen that could not be classified as Type I collagen (i.e., 2 $\alpha 1$ (I) > $\alpha 2$) was designated "other" collagen (Tables I and II). The amount of "other" collagen synthesized by normal and rheumatoid synovial cells varied from 0 to 15% by SDS–PAGE analysis. Estimations of "other" collagen by CMC chromatography varied from 0 to 30%. As observed with the SDS–PAGE separation, osteoarthritic synovial cells produced the highest percentages of "other" collagen (Table II).

Discussion. The data reported here establish that human synovial cells express a significant commitment and potential for the synthesis of Type III collagen in both primary and daughter cell cultures which can be detected by the application of two separation techniques. The amount of Type III collagen synthesized by normal synovial cells in culture varied from 10 to 17%; this is comparable to the 10–20% Type III collagen recovered from normal mammalian skin (22). Normal synovial cells obtained from a 9-year-old patient synthesized 38% Type III collagen (Fig. 3), which may reflect an increased rate of synthesis at an early age. Fetal human skin has been reported to have a much higher percentage of extractable Type III collagen than adult skin (23).

In this study, three out of four cultures observed to be producing the highest percentage of Type III collagen at the first passage were derived from patients with rheumatoid arthritis (if the nonrheumatoid 9-year-old patient is not considered). However, the mean values obtained for rheumatoid cultures at the first passage were not significantly different to the nonrheumatoid controls. Rheumatoid synovial cells at the fourth passage were found to synthesize a significantly ($P < 0.05$) greater percentage of Type III collagen when compared to nonrheumatoid synovial cells. However, only a limited number of patients were investigated and additional studies will be necessary to confirm the biological significance of this observation.

The monolayer cultures used in this study contained a heterogeneous population of cells similar to the synovial cell cultures described by Marsh *et al.* (24). The variability of the percentage of Type III collagen synthesized by rheumatoid and nonrheumatoid cultures may reflect differences in cell populations from one culture to another. Hance and Crystal (25) have reported that the ratio of Type I to Type III collagen, synthesized by cultures of human lung fibroblasts, is independent of cell density and passage number. Further, the studies reported here were all conducted on cells at confluence. Hence the significant difference observed between rheumatoid and nonrheumatoid cultures at the fourth passage may be due to the selection of a certain cell type that is present in rheumatoid cultures and predominantly synthesizes Type III collagen. Although this heterogeneity of cell population creates interpretation problems, it is proposed that at present it is a valid and appropriate system for studies on the biology and pathophysiology of the synovium. If the suggestion of Weiss *et al.* (11) that the percentage of Type III collagen reflects the activity or stage of development of the rheumatoid pannus is correct, the design of future studies should include an assessment of the degree of active synovial cell proliferation at the time specimens are obtained as a result of reconstructive surgery.

The electrophoretic separation used in this study for the quantitation of the rate of synthesis of Types I and III collagen is reproducible with a high degree of accuracy in duplicate samples. The relative rapidity of the gel electrophoresis separation, and the requirement for smaller amounts of radioactivity (both compared to CMC chromatography) are conditions required for the application of this separation technique to the screening of the large number of samples characteristically generated in a clinical study. The significant parallelism in the ordering of values when comparing the electrophoresis system to the CMC chromatography further confirms that the separation systems used here can be expected to provide a reproducible estimation of relative changes in the percentage of Type III collagen produced by these human cell culture systems.

In the present study, collagen that could not be designated as Type I or Type III, but which migrated on electrophoresis or eluted from CMC as α1 (I) is referred to as "other" collagen. "Other" collagen may signify the production of Type I trimer (α, $[I]_3$ chains) which has previously been reported in the literature (9, 26). However, the collagen migrating in the α1 (I) region during SDS–PAGE may include α[A] chains (8) and also some reduced α1 (III) chains that should have migrated as unreduced high-molecular-weight trimers. Specific loss of α2 chains to the CMC column could account for high α1 (I)/α2 ratios in this system. Thus the significance of the finding that the percentage of "other" collagen synthesized by osteoarthritic synovial cells is greater than normal or rheumatoid synovial cells remains unclear at this time. Their characterization may be important in view of the influence of αA and αB on cell migration (4).

Our findings that rheumatoid synovial tissue contains a high proportion of extractable collagen and that a significant proportion is Type III agree with the earlier work of others (11, 12). The fact that osteoarthritic tissue contains relatively little extractable collagen would indicate that it is similar to normal synovial tissue in this regard. Since we have found that synovial cell cultures grown from normal, osteoarthritic, and rheumatoid tissue all synthesize similar ratios of Type III to Type I collagen, our work supports the view of Eyre and Muir (12) that the proportion of Type III to Type I collagen is similar in normal and rheumatoid synovial tissue, and the work of Vuorio (27), who found that the proportion of Type I and Type III collagen produced by cultured normal and rheumatoid cells were similar. However, the possibility still exists that synovial cell Type III collagen synthesis may be elevated during the acute proliferative stage due to the presence of local inflammatory mediators released within the diseased joint. These could enhance Type III collagen synthesis in synovial tissue *in vivo*, but in tissue culture in the absence of such factors, synthesis of

Type III collagen returns to normal. This would explain our observation that primary organ cultures of rheumatoid synovial tissue fragments synthesize a higher percentage of Type III collagen than do rheumatoid cells grown from explant cultures.

It has been reported that granulocyte collagenase preferentially breaks down Type I rather than Type III collagen and this could also lead to a build up of Type III collagen within the inflamed synovium (28), especially under the conditions of elevated collagen metabolism that occur in rheumatoid tissue. Increased amounts of Type III collagen in human synovium, as a result of either enhanced synthesis or reduced degradation, could perpetuate the rheumatoid disease process. Studies of humoral and cell-mediated immune activity of peripheral lymphocytes obtained from patients with rheumatoid arthritis have demonstrated reactivity to Type III collagen comparable to that found against Type II collagen of cartilage (29, 30). These studies and the demonstration by Golds *et al.* (31) that collagen peptides can stimulate rheumatoid lymphocytes, which in turn can enhance collagenase secretion by synovial cells, suggest that the interaction of the immunoinflammatory, degradative, and proliferative components of the rheumatoid lesion may be interrelated and amenable to study in a tissue culture system as described here. Recent studies in our laboratory indicate that pharmacological concentrations of gold sodium thiomalate result in a change (decrease) in the percentage of Type III collagen synthesized by rheumatoid synovial cells in culture (19). This drug-induced change in the percentage of Type III collagen produced also identifies therapeutic implications which can be derived from these investigations.

1. Harris, E. D., Arthritis Rheum. **19**, 68 (1976).
2. Kleinman, H. K., McGoodwin, E. B., Martin, G. R., Klebe, R. J., Fietzek, P. P., and Woolley, D. E., J. Biol. Chem. **253**, 5642 (1978).
3. Chiang, T. M., Postlewaite, A. E., Beachey, E. H., Seyer, J. M., and Kang, A. H., J. Clin. Invest. **62**, 916 (1978).
4. Stenn, K. S., Madri, J. A., and Roll, F. J., Nature (London) **277**, 229 (1979).
5. Liotta, L. A., Vembu, D., Kleinman, H. K., Martin, G. R., and Boone, C. Nature (London) **272**, 622 (1978).
6. Prockop. D. J., Kivirikko, K. I., Tuderman, L., and Guzman, N. A., N. Engl. J. Med. **301**, 13, 77 (1979).
7. Rhodes, R. K., and Miller, E. J., Biochemistry **17**, 3443 1978.
8. Brown, R. A., Shuttleworth, C. A., and Weiss, J. B. Biochem. Biophys. Res. Commun. **80**, 866 (1978).
9. Benya, P. D., Padilla, S. R. and Nimni, M. E., Biochemistry **16**, 865 (1977).
10. Bailey, A. J., Sims, T. J., Lelous, M., and Bazin, S., Biochem. Biophys. Res. Commun. **66**, 1160 (1975).
11. Weiss, J. B., Shuttleworth, C. A., Brown, R., Sedowfia, K., Baildam, A., and Hunter, J. A., Biochem. Biophys. Res. Commun. **65**, 907 (1975).
12. Eyre, D. R. and Muir, H., Connect. Tissue Res. **4**, 11 (1975).
13. Kellgren, J. H., and Lawrence, J. S., Ann. Rheum. Dis. **16**, 494 (1957).
14. Kuttan, R., Parrott, D. P., Kaplan, S. R., and Fuller, G. C., Res. Commun. Chem. Pathol. Pharmacol. **26**, 337 (1979).
15. Goldberg, R. L., and Fuller, G. C., Anal. Biochem. **90**, 69 (1978).
16. Kivirikko, K. I., Laitinen, O., and Prockop, D. J., Anal. Biochem. **19**, 249 1967.
17. Furthmayr, H., and Timpl, R., Anal. Biochem. **41**, 510 1971.
18. Neville, D. M., J. Biol. Chem. **246**, 6328 (1971).
19. Goldberg, R. L., Parrott, D. P., Kaplan, S. R., and Fuller, G. C., Biochem Pharmacol. **29**, 869 (1980).
20. Fujii, T., and Kuhn, K., Physiol. Chem. **356**, 1793 (1975).
21. Miller, E. J., *in* "The Methodology of Connective Tissue Research" (D. A. Hall, ed.), p. 197. Joynson–Bruvers, Oxford (1976).
22. Miller, E. J., Mol. Cell. Biochem. **13**: 165 (1976).
23. Epstein, E. H., J. Biol. Chem. **356**, 1793 (1974).
24. Marsh, J. M., Maini, R. N., Weibkin, O. W., and Dumonde, D. C., J. Rheumatol. **5**, 148 1978.
25. Hance, A. J., and Crystal, R. G., Nature (London) **268**, 152 (1977).
26. Narayanan, A. S., Page, R. C., and Kuzan, F., Lab. Invest. **39**, 61 (1978).
27. Vuorio, E., Scand. J. Clin. Lab. Invest. **37** (Suppl. 149), (1977).
28. Horwitz, A. L., Hance, A. J., and Crystal, R. G., Proc. Nat. Acad. Sci. (USA) **74**, 897 (1977).
29. Andriopoulos, N. A., Mestacky, J., Miller, E. J., and Bradley, E. L., Arthritis Rheum. **19**, 613 (1976).
30. Stuart, J. M., Postlethwaite, A. E., Kang, A. H., and Townes, A. S., Arthritis Rheum. **22**, 665 (1979).
31. Golds, E. E., Lyons, H., Cooke, T. D. V., Van der Rest, M., Poole, A. R., Arthritis Rheum. **22**, 614 (1979).

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