Leukocyte-Connective Tissue Cell Interaction. I. Stimulation of Hyaluronate Synthesis by Live and Dead Leukocytes

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During the interaction of fibroblasts and leukocytes in vitro, there was a two- to sixfold increase in the total amount of hyaluronate formed, and a corresponding increase in the rate (pg./cell/day) of hyaluronate synthesis. Increased glucose uptake from the culture medium (two- to fourfold) was associated with stimulation of hyaluronate formation. Cultures receiving leukocytes suffered an 8 to 33 per cent decrement in the fibroblast population. Leukocytes themselves consumed little glucose and produced no detectable hyaluronate. Viable and nonviable lymphocytes and PMN's induced qualitatively similar behavioral alterations in synovial fibroblasts. Fibroblasts from normal and rheumatoid joints responded similarly to WBC. Normal and rheumatoid WBC had similar potential for altering the behavior of fibroblasts in vitro. Erythrocytes had little effect on fibroblast cultures.

OVERPRODUCTION of joint fluid hyaluronic acid by rheumatoid synovial tissue can be visualized as a consequence of altered synthetic activity of specialized connective tissue cells functioning in an abnormal milieu of infiltrating lymphoid cells and polymorphonuclear leukocytes (PMN). To explore the nature of possible interactions between such diverse cell types, we established monolayer cultures of human fibroblasts from normal and rheumatoid articular tissue and incubated these cultures with isolated leukocytes. The peripheral blood leukocytes were isolated from normal persons and rheumatoid patients, and the lymphocyte-rich fractions and PMN-rich fractions which were prepared were added to monolayer fibroblast cultures to simulate the cell mixtures found in the inflamed synovial membrane.

Specific cellular interactions have been considered important in directing the sequences of embryonic development, and the functional performance of corneal epithelium and the cells in the underlying stroma are known to be interdependent.[1] The possibility that abnormal connective tissue cell function might be induced by inflammatory cells draws some support from studies demonstrating damage to fibroblast cultures caused by addition of nonimmune lymphoid cells.[2] Both phytohemagglutinin and streptolysin accelerated the capacity of lymphoid cells to cause plaques (i.e., a visible lesion in the fibroblast monolayer associated with cell destruction), and irradiation, cortisone, and chloroquine did not suppress plaque formation in these mixed cultures. One study indicated that lymphoid cells from patients

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with connective tissue diseases were more cytotoxic than those from normal persons.[3]

Materials and Methods

Fibroblast cultures were derived from synovial tissue obtained at arthrotomy or amputation and were grown as monolayers in serum dilution bottles as previously reported.[4-6] Cells were nourished by a semisynthetic medium consisting of 80 per cent medium 1066, 10 per cent fetal calf serum, and 10 per cent pooled, heat inactivated human serum supplemented with L-glutamine. Penicillin and streptomycin sulfate (100 μg./ml.) were added to the culture medium.

Separation of lymphocytes from peripheral blood was accomplished by the procedure of Coulson and Chalmers.[7] Freshly drawn blood was defibrinated with glass beads* in an Erlenmeyer flask by rotating and shaking for 5 min. Defibrinated blood was added to a siliconized prescription bottle, and one part of a 3 per cent solution of gelatin† in 0.15 *M* NaCl was added to three parts of blood. The gelatin solution was sterilized with a Seitz filter and used fresh at a temperature of 37°C. The gelatin-blood mixture was incubated for 30 min. at 37°C, and the supernatant portion was removed. The supernatant fluid was centrifuged at 200 × *g* for 10 min. to sediment the lymphocytes, which were recovered with a yield ranging from 15–50 per cent of those in the whole blood. Lymphocytes accounted for 70–98 per cent of the separated leukocytes. Erythrocytes were always present in varying concentrations. These lymphocytes appeared viable in that they excluded 0.4 per cent erythrosine B.[8] When phytohemagglutinin was added to such lymphocyte preparations, the transformation rate was 70–90 per cent and 1–7 per cent of the cells were in mitosis. Evidence of transformation was sought after 3 days' incubation and 4–6 hours after the addition of colchicine.

Polymorphonuclear leukocytes were separated from peripheral blood using a modification of the method described by Rabinowitz.[9] Fresh blood was drawn in a plastic syringe containing 500 units of preservative-free heparin for each milliliter of blood. The heparinized blood was sedimented in the syringe for 1 hour at 37°C and the supernatant suspension of leukocytes in plasma was transferred to a prescription bottle, using a bent needle to avoid inverting the syringe. Prescription bottles were previously washed with concentrated nitric acid, exhaustively rinsed with double distilled water, and siliconized. Medium consisting of 80 per cent medium 1066 and 20 per cent human serum, supplemented with L-glutamine, penicillin, and streptomycin in a volume equal to ⅝ that of the plasma, was added to the prescription bottle and the mixture incubated at 37°C for 30 min. After incubation the supernatant fluid was discarded, leaving a visible layer of cells attached to the floor of the flask. A solution of buffered saline with EDTA was added immediately, and the prescription flask was gently shaken for 10 min. at room temperature while the clear saline solution became increasingly cloudy as cells detached from the glass. To wash the leukocytes from 60.0 ml. of blood, 40 ml. of the buffered saline with EDTA was used.* The cell suspension was centrifuged at 200 × *g* for 10 min. at room temperature. Staining with erythrosine B showed 100 per cent viability (e.g., dye exclusion). Polymorphonuclear leukocytes obtained by this method exhibited active ameboid motion when planted on fibroblasts in tissue culture flasks, and Wright stains showed preservation of cellular structure and cytoplasmic granulation. The yield ranged between 20 and 30 per cent of the initial number of PMN cells in the blood drawn and a purity of 70–95 per cent, the contaminants being primarily mononuclear cells with a few erythrocytes.

Phytohemagglutinin (PHA) used in the interaction experiments was phytohemagglutinin-P,† and 0.02 ml. of phytohemagglutinin (from the 5 ml. in the original container) was added to each 10 ml. of medium. The dry PHA was diluted with hemagglutinination buffer.†

Replicate cultures of fibroblasts were planted in either T–30 flasks or serum dilution bottles. The inoculum in different experiments ranged from 0.4 to 1.0 × 10^6, and each experimental group in the protocol contained three flasks. Towards the end of the fibroblast growth cycle, 5.0 × 10^6 washed leukocytes were added to the fibroblast cultures along with complete medium replacement. Except where specifically noted, lymphocytes were incubated for 3 days with fibroblast monolayers and polymorphonuclear leukocytes for 2 days. At the end of the 7 day growth cycle, the cultures were separated from the glass with trypsin and the resulting cell suspension enumerated and sized using a Coulter model B cell counter with the lower

*Clear glass beads, 5 mm. in diameter, from Matheson Company.

†Baker and Adamson, code number 1797.

*Buffered saline with EDTA was made as follows: disodium EDTA, 0.2 Gm.; NaCl, 8.0 Gm.; KCl, 0.2 Gm.; Na_2HPO_4, 1.15 Gm.; KH_2PO_4, 0.2 Gm.; glucose, 0.2 Gm.; and water, 1000 ml.

†Difco Laboratories, Detroit, Mich.

Table 1.—Effects of Viable Polymorphonuclear Leukocytes on "Rheumatoid" Fibroblasts

Fibroblast strain*	Diagnosis	Leukocyte† additive	Final fibroblast count × 10^6	MCV,‡ μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
P.G.–D, 1	RA	None	1.533	2400	0.42	40.0	13.0
P.G.–D, 2	RA	None	1.841	2530	0.32	51.6	14.0
P.G.–D, 3	RA	None	1.888	2450	0.30	48.0	12.7
Average	—	—	1.754	2493	0.34	46.5	13.2
P.G.–D, 4	RA	PMN_{RA}	1.473	3100	1.49	283.0	96.1
P.G.–D, 5	RA	PMN_{RA}	1.675	2980	1.31	189.0	56.4
P.G.–D, 6	RA	PMN_{RA}	1.417	2990	1.55	245.0	86.5
Average	—	—	1.522	3023	1.45	239.0	79.7
P.G.–D, 7	RA	PMN_N	1.250	2890	1.76	267.0	106.8
P.G.–D, 8	RA	PMN_N	1.722	2890	1.28	270.0	78.4
P.G.–D, 9	RA	PMN_N	1.733	2890	1.15	260.0	75.0
Average	—	—	1.565	2890	1.40	266.0	86.7

Fibroblast inoculum = 0.4×10^6/flask.

* P.G. refers to the initials of the donor, while the D indicates the number of times (4) the strain has been subcultured. Individual flasks were numbered serially.

† Polymorphonuclear leukocyte inocula, both normal (PMN_N) and rheumatoid (PMN_{RA}), were 5.0×10^6 cells/flask. Purity of the PMN preparation was 95%. The "interaction" period for the two kinds of cells together was the last 2 days of a 7 day growth cycle.

‡ MCV = mean cell volume.

threshold set to exclude blood cells and debris. Viability of the fibroblast suspension was estimated by the erythrosine B exclusion method.[8] The residual glucose in culture media was measured using a glucose oxidase method.[10] Hyaluronic acid in culture media was measured by a method published earlier from this laboratory wherein the mucopolysaccharide is isolated and its uronic acid content determined.[11] The detailed format of individual experiments can be best appreciated from the tables.

Glucose uptake was calculated from the following relation:

$$\text{mg. glucose}/10^6 \text{ cells/day} = \frac{C_o - C_i}{T \times \text{cell count}}$$

where C_o = mg. glucose in unused medium, C_i = residual glucose in medium from the final medium change in the culture cycle, T = time in days that last medium change was in contact with the culture, and the cell count is expressed in units of 1 million. The hyaluronic acid synthesis rate is also calculated from the hyaluronic acid measurement in the last medium change and the total fibroblast count, using this relation:

$$\text{pg. HA/cell/day} = \frac{\mu\text{g. HA}}{T \times \text{cell count}}$$

where T = time in days during which final medium change was in contact with the cells, and the cell count refers to the total number present in the flask. As an example, the data concerning culture PG–D_2, flask 1 (Table 1), were calculated as follows:

$$\text{HA synthesis rate} = \frac{40\ \mu\text{g.}}{2 \text{ days} \times 1.533 \times 10^6} = 13.0 \text{ pg./cell/day}$$

Results

Synovial fibroblasts from an elbow of a patient (P.G.) with rheumatoid arthritis produced 6.0 times more hyaluronic acid per cell per day than control cultures when interacted with allogeneic PMN_{RA}, and 6.6 times more when interacted with allogeneic PMN_N (Table 1). There was a corresponding increase in glucose consumption when compared to controls. Polymorphonuclear leukocytes from both sources induced a minor decrease in the mean total cell count.

When the same strain of fibroblasts was incubated with lymphocytes in a separate experiment (Table 2), the increase in

Table 2.—Effects of Viable Lymphocytes* on "Rheumatoid" Fibroblasts†

Fibroblast strain	Diagnosis	Leukocyte additive	Final fibroblast count $\times 10^6$	MCV, μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
P.G.–D	RA	None	2.265 (1.794–2.560)	1743 (1720–1770)	0.13 (0.08–0.19)	75.6 (74.0–77.0)	11.6 (9.5–14.3)
P.G.–D	RA	L_{RA}	2.077 (1.920–2.180)	2370 (2310–2450)	0.60 (0.56–0.66)	298.3 (286.0–318.0)	48.0 (44.8–50.5)
P.G.–D	RA	L_N	2.066 (1.948–2.280)	2150 (2070–2250)	0.61 (0.56–0.65)	350.8 (340.0–368.4)	56.5 (53.8–58.9)
P.G.–D	RA	PHA‡	1.701 (1.502–1.839)	2186 (2160–2200)	0.19 (0.15–0.27)	94.0 (92.0–96.0)	18.5 (17.0–20.4)
P.G.–D	RA	L_{RA}* PHA	0.939 (0.770–1.201)	2180 (2100–2310)	1.21 (0.93–1.45)	234.6 (216–264)	84.9 (78.3–87.9)
P.G.–D	RA	L_N* PHA	1.177 (1.086–1.232)	2210 (2150–2280)	1.08 (1.03–1.17)	212.6 (200–222)	60.1 (53.0–67.5)

* Lymphocytes from normal persons (L_N) and rheumatoid patients (L_{RA}) were added in a concentration of 5.0×10^6 cells, 80% purity. The fibroblast inoculum was 0.6×10^6 cells/flask. The "interaction" time was the final 3 days of a 7 day growth cycle.

† The numbers presented include the mean values of observations (or derived numbers), and the figures in parentheses are the absolute range of the triplicate observations, or derived numbers.

‡ Phytohemagglutinin-P was used in a concentration of 0.02 ml. per 10 ml. of complete culture medium.

Table 3.—Effects of Viable Polymorphonuclear Leukocytes on "Normal" Fibroblasts

Fibroblast strain	Diagnosis	Leukocyte additive	Final fibroblast count $\times 10^6$	MCV, μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
D.M.–I	Normal	None	2.44 (2.36–2.54)	2800 (2800–2800)	0.95 (0.89–1.01)	309.4 (302.0–316.8)	64.9 (62.4–67.5)
D.M.–I	Normal	PMN_{RA}	2.28 (2.18–2.38)	3680 (2560–3800)	1.05 (1.00–1.09)	352.6 (350.4–357.6)	77.8 (73.6–82.0)
D.M.–I	Normal	PMN_N	2.21 (2.16–2.28)	3610 (3550–3680)	1.07 (1.03–1.10)	331.2 (327.4–338.4)	74.8 (74.2–75.9)

Fibroblast inoculum was 0.4×10^6/flask, PMN_{RA} inoculum was 4.6×10^6 cells/flask (70% purity), and PMN_N inoculum was 5.0×10^6 cells/flask (80% purity). The "interaction" time was 2 days.

hyaluronate production per cell per day was over four times the control value. There was a marked increase in glucose uptake in both groups, similar to that noticed in the PMN experiment. A modest decrease in the final number of lymphocyte-treated fibroblasts was noted, also similar in magnitude to that seen with PMN leukocytes. The viability of the fibroblasts was not significantly affected by interaction with blood cells, since over 90 per cent excluded erythrosine B.

"Normal" synovial fibroblasts obtained from the wrist of a young woman (D.M.) who had had surgery for a congenital malformation were interacted with allogeneic PMN_{RA} and PMN_N. In this experiment (Table 3), only modest stimulation of the specific rate of hyaluronate synthesis was noted. A similar increase was noted on a second occasion (Table 4). On the other hand, when the same strain of fibroblasts was incubated with different allogeneic PMN_{RA} on another occasion (Table 5), they produced four times more hyaluronate per cell per day than the controls. It is of interest that on both occasions when the increase in hyaluronate production by the interacted cells was minimal, the "basal" rate of hyaluronate production of control cultures was high (compared with the control in Table 5). In most experiments, an increase of the mean corpuscular volume of the interacted fibroblasts was recorded.

Table 4.—Comparison of the Effects of Viable and Nonviable Polymorphonuclear Leukocytes on Normal Fibroblasts

Fibroblast strain	Diagnosis	Leukocyte additive	Final fibroblast count × 10^6	MCV, μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
D.M.–G	Normal	None	4.47	2316	0.36	354.0	39.6
			(4.36–4.64)	(2250–2350)	(0.35–0.37)	(334.0–392.0)	(36.0–44.5)
D.M.–G	Normal	Viable PMN_N	3.17	3150	0.60	296.6	46.8
			(3.00–3.28)	(3150–3150)	(0.58–0.63)	(288.0–302.0)	(43.9–50.0)
D.M.–G	Normal	Nonviable PMN_N	4.21	2296	0.41	386.6	45.9
			(4.12–4.34)	(2290–2300)	(0.41–0.42)	(360.0–424.0)	(43.7–48.8)

Fibroblast inoculum was 0.86 × 10^6/flask; PMN_N inoculum was 5.0 × 10^6, 78% purity. The "interaction" time was the final 2 days of a 7 day growth cycle.

Table 5.—Comparison of Effects of Viable and Nonviable Leukocyte Mixtures on Normal Fibroblasts

Fibroblast strain	Diagnosis	Leukocyte additive	Final fibroblast count × 10^6	MCV, μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
D.M.–F	Normal	None	4.31	2717	0.15	194.0	15.0
			(4.04–4.60)	(2650–2800)	(0.14–0.16)	(174–224)	(14.5–16.2)
D.M.–F	Normal	Viable $leuk_{RA}$	3.16	2893	0.40	514.7	54.3
			(2.86–3.46)	(2800–2980)	(0.37–0.44)	(464–544)	(52.4–56.5)
D.M.–F	Normal	Nonviable $leuk_{RA}$	3.38	2750	0.37	685.3	68.0
			(3.00–3.84)	(2750–2750)	(0.33–0.42)	(632–744)	(63.8–75.6)
D.M.–F	Normal	Viable PMN_{RA}	2.66		0.47	507.0	63.7
			(2.50–2.85)		(0.43–0.51)	(488–534)	(62.5–65.1)

The preparation $leuk_{RA}$ contained 50% lymphocytes; the remainder were mostly PMN. The inoculum of $leuk_{RA}$ was 10.0 × 10^6. The inoculum of viable PMN_{RA} was 4.6 × 10^6 cells/flask (90%); the fibroblast inoculum was 0.65 × 10^6/flask. The "interaction" time was the final 3 days of a 7 day growth cycle.

Table 6.—Effects of Viable Lymphocytes on Normal Fibroblasts

Fibroblast strain	Diagnosis	Leukocyte additive	Final fibroblast count × 10^6	MCV, μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
D.M.–G	Normal	None	3.793	2307	0.15	328.3	29.3
			(3.260–4.200)	(2300–2320)	(0.15–0.16)	(301.0–340.0)	(23.9–34.8)
D.M.–G	Normal	L_{RA}	2.527	2467	0.59	703.3	94.7
			(2.260–2.960)	(2430–2520)	(0.49–0.65)	(656.0–750.4)	(73.9–110.7)
D.M.–G	Normal	L_N	3.360	2427	0.44	866.6	86.4
			(2.980–3.560)	(2400–2460)	(0.41–0.49)	(832.0–912.0)	(80.6–93.1)

The fibroblast inoculum was 0.4 × 10^6, L_{RA} inoculum was 4.6 × 10^6 cells/flask (70% purity), and the L_N inoculum was 5.0 × 10^6 cells/flask (80% purity). The "interaction" interval was the final 2 days of a 7 day culture cycle.

When normal fibroblasts (D.M.) were incubated with allogeneic L_{RA} and L_N (Table 6), hyaluronate production increased about threefold. A minor increase in the mean cell volume was seen. Glucose uptake per flask was three to four times control values for L_N and L_{RA} stimulated cultures.

Mixtures of viable WBC incubated with "normal" fibroblasts (D.M.) (Tables 5 and 7) showed a large increase in hyaluronate formation. Erythrocytes, even when added in very large numbers, had virtually no effect on the parameters measured (Table 7).

The Effect of Frozen-Thawed Leukocytes

To determine whether viability of leukocytes was required to induce altered con-

Table 7.—Comparison of the Effects of Viable Leukocytes and Erythrocytes on Normal Fibroblasts

Fibroblast strain	Diagnosis	Leukocyte additive	Final fibroblast count $\times 10^6$	MCV, μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
D.M.-G	Normal	None	5.06 (4.92–5.24)	2096 (2050–2120)	0.05 (0.05–0.06)	138.0 (137–140)	9.1 (8.8–9.2)
D.M.-G	Normal	PMN_N	3.87 (3.80–3.96)	2333 (2300–2400)	0.30 (0.30–0.30)	512.7 (492–524)	44.2 (45.1–46.0)
D.M.-G	Normal	$leuk_N$	4.07 (4.02–4.12)	2300 (2300–2300)	0.28 (0.28–0.29)	538.0 (508–568)	44.1 (42.1–46.0)
D.M.-G	Normal	$erythro_N$	5.31 (5.14–5.48)	2140 (2080–2200)	0.07 (0.07–0.07)	159.5 (155–164)	10.0 (10.00–10.1)

The fibroblast inoculum was 1.0×10^6/flask; PMN_N inoculum was 5.0×10^6 (95% purity); $leuk_N$ inoculum was 5.0×10^6 (45% lymphocytes); erythrocyte ($erythro_N$) inoculum was 35.0×10^6/flask. The "interaction" time was the final 3 days of a 7 day growth cycle.

nective tissue cell behavior, we subjected equivalent numbers of leukocytes to three cycles of freeze-thawing and added the cell debris to fibroblast cultures, with the results summarized in Tables 4, 5, 7, and 8. These data indicate that dead lymphocytes and, to a lesser degree, dead polymorphonuclear leukocytes, both separately and together, alter fibroblast function in the same directions and nearly to the same degree as their live counterparts. Where glucose uptake by 5.0×10^6 viable leukocytes was measured in the absence of fibroblasts, consumption was only about 1/5 that seen in control fibroblast cultures. These data suggest that the added leukocytes themselves play little part in the increased glucose consumption, but that they accelerate fibroblast utilization of glucose concurrent with the outpouring of fibroblast-formed hyaluronate.

The Effect of Lymphocyte "Activation" on "Interaction" Phenomena

In the presence of PHA (Table 2), lymphocytes were adherent to the fibroblastic monolayer after about 1½ hours, and after 3 days approximately 50 per cent of them were still attached to the fibroblasts while the remainder floated in clumps. In the absence of PHA, most of the lymphocytes floated or were loosely attached to the fibroblast monolayer. The presence of PHA alone caused a 25 per cent decrease in the final cell count as compared to the controls, while the addition of rheumatoid lymphocytes and normal lymphocytes in the presence of PHA caused a 55 and 40 per cent decrease, respectively. All the additives in this experiment caused a similar increase in the mean corpuscular volume of "interacted" fibroblasts. The glucose uptake was only slightly increased by PHA alone, while it was significantly increased by the addition of lymphocytes alone or by both lymphocytes and PHA. There was a minimal increase in the formation of hyaluronic acid when only PHA was present in the medium, while there was a large increase in the production of hyaluronic acid when the additive included lymphocytes.

Identification of Culture-Produced Hyaluronate

By virtue of the analytic procedure for measuring culture-produced acid mucopolysaccharides, it was clear that synovial fibroblasts added a cetylpyridinium chloride-precipitable macromolecule containing uronic acid to the culture medium.[11] While this has been adequately characterized as hyaluronic acid in media from typical synovial cultures,[4] it seemed prudent to characterize the acid mucopolysaccharide (MPS) from leukocyte-treated cultures. Viscometric measurements controlled with

Table 8.—Effects of Viable and Nonviable Lymphocytes on Normal Fibroblasts

Fibroblast strain	Diagnosis	Leukocyte additive	Final fibroblast count × 10^6	MCV, μ^3	Glucose uptake, mg./10^6 cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
Wa.K.–I	Normal	None	6.10 (5.65–6.47)	1800 (1800–1800)	0.16 (0.15–0.19)	160 (152–168)	13.2 (12.3–14.9)
Wa.K.–I	Normal	Viable L_N	4.89 (4.56–5.52)	1567 (1500–1600)	0.65 (0.56–0.70)	506.7 (488–544)	52.4 (44.2–59.6)
Wa.K.–I	Normal	Nonviable L_N	5.12 (4.76–5.45)	1800 (1700–1900)	0.48 (0.44–0.50)	439.3 (352.0–492.0)	41.9 (37.0–45.3)

The fibroblast inoculum was 1.06×10^6/flask; L_N inoculum was 5.0×10^6/flask (80% purity). The "interaction" time was the final 2 days of a 7 day culture cycle.

Table 9.—Characterization of Isolated Culture-Produced Hyaluronate (HA)

Measurement on isolated HA	Control cultures	Cultures + L_{RA}	Cultures + PMN_{RA}	Method, ref. no.
Hexosamine	Glucosamine	Glucosamine	Glucosamine	12
Hexosamine: hexuronic acid ratio	0.73	0.84	0.82	13, 14
Carbazole: orcinol ratio	1.09	1.18	0.90	15, 16
CSC of CPC Complex*	0.1 *M* $MgCl_2$	0.1 *M* $MgCl_2$	0.1 *M* $MgCl_2$	17
Protein, %	5.7	4.4	7.4	18

* CSC refers to the initial salt concentration required to prevent flocculation of a mucopolysaccharide-cetylpyridinium complex in aqueous solution.

testicular hyaluronidase[6] made it clear that the culture-produced mucopolysaccharide was hyaluronidase-labile and of high molecular weight ($[\eta] = 15$–18). Medium samples from control, PMN, and lymphocyte-treated cultures were subjected to proteolytic digestion (pronase); the mucopolysaccharide was then isolated with cetylpyridinium chloride and converted to the sodium salt. As shown in Table 9, the mucopolysaccharide preparations were essentially protein-free and exhibited a critical salt concentration for the cetylpyridinium chloride-MPS complex typical of hyaluronate.[17] In addition, the carbazole:orcinol ratio was typical of hyaluronate (approximately 1.0). Hydrolysis of aliquots of isolated MPS permitted measurement and identification of the hexosamine moiety, which was entirely glucosamine. In summary, the leukocyte-treated cultures made the same MPS (hyaluronate) which is characteristic of control synovial cultures; thus, the leukocyte-induced alteration in mucopolysaccharide metabolism appears to be entirely quantitative in this model system.

Discussion

These experiments demonstrated that both viable and disrupted leukocytes induced overproduction of hyaluronic acid by fibroblasts when the two types of cells were associated in vitro. Both lymphocytes and polymorphonuclear leukocytes had this stimulatory effect on connective tissue cells, and neither produced measureable hyaluronic acid when cultured alone.

Recent evidence indicates that human leukocytes contain and may synthesize small amounts of acid mucopolysaccharide thought to be chondroitin sulfate.[19] Since these authors needed 100 times as many leukocytes as we used, in addition to sensitive tracer methods, it is not surprising that our chemical analyses of leukocytes in the numbers used in our experiments disclosed no significant mucopolysaccharide uronic acid contribution from that source.

Analysis of the increased mucopolysaccharide formed under the leukocyte influence showed it to be hyaluronic acid, the product normally formed by fibroblasts of synovial origin. It appeared to make little

difference whether the leukocytes came from normal persons or patients with rheumatoid arthritis. In the same manner, both "normal" and "rheumatoid" fibroblasts were capable of responding to the leukocyte influence.

Accelerated glucose disappearance also occurred during the cellular interaction, and the magnitude of the increment in glucose uptake seemed to parallel the increased hyaluronate formation. Accelerated glucose disappearance was always accompanied by a rapid decrease in medium pH. Since leukocytes alone removed little medium glucose, and since disrupted leukocytes also caused increased glucose disappearance, it is reasonable to conclude that the leukocytes caused the fibroblasts to remove increased amounts of glucose from the medium.

Under the conditions of these experiments, leukocytes usually induced a modest reduction in the fibroblast population. Other investigators, using microscopic methods, have described a toxic effect of lymphocytes on fibroblasts in tissue culture.[2,3] The present experiments provide quantitative support for such observations and suggest that this "toxic' effect may be nonspecific, perhaps related to profound depletion of medium glucose or to hydrogen ion excess.

Phytohemagglutinin caused tight adherence of the lymphocytes to the fibroblastic monolayer and increased toxicity to the fibroblasts. Lymphocytes have previously been shown to be toxic to fibroblasts and other target cells in the presence of phytohemagglutinin.[20,21] In the present experiments, this toxicity, measured by fibroblast counts, was more profound when rheumatoid lymphocytes were the "attacking" cells. Whether this would be generally true for rheumatoid lymphocytes is unknown. Since Nowell's initial report[22] showing the ability of phytohemagglutinin to induce "blastoid" transformation and mitosis in lymphocytes, subsequent reports have shown that the phytohemagglutinin may increase the antibody production of the lymphocyte,[23] and one of these reports[24] suggests that lymphocytes from a patient with acute rheumatoid arthritis in the presence of PHA showed more stimulation of globulin synthesis than lymphocytes from normal persons. The present experiments demonstrated a toxic effect of phytohemagglutinin alone on the fibroblastic monolayer, causing decrease in the fibroblast count, the phytohemagglutinin alone being more toxic in this respect than lymphocytes alone. Interaction experiments carried out in the presence of phytohemagglutinin must be considered in the light of demonstrated effects of the mitogenic agent alone on the target fibroblasts. Since the PHA effects were capricious, varying with the concentration, age, and particular batch of PHA, we found experiments which included this agent difficult to interpret, and for this reason chose to emphasize the results of experiments where leukocytes and connective tissue cells were interacted in the absence of this complicating additive.

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SUMMARIO IN INTERLINGUA

Durante le interaction de fibroblastos e leucocytos observate in vitro, un duple a sextuple augmento esseva notate in le quantitate total del hyaluronato formate insimul con un correspondente augmento in le prorata (pg/cellula/die) del synthese de hyaluronato. Le augmentate acceptation de glucosa ab le medio cultural (per un factor de 2 a 4) esseva associate con stimulation del formation de hyaluronato. Culturas recipiente leucocytos suffreva un declino per 8 a 33 pro cento in le population de fibroblastos. Le leucocytos como tal consumeva pauc glucosa e produceva nulle detegibile hyaluronato. Viabile e nonviabile lymphocytos e neutrophilos polymorphonucleari

induceva qualitativemente simile alterationes del comportamento de fibroblastos synovial. Fibroblastos ab articulationes normal e rheumatoide respondeva similemente a leucocytos. Leucocytos normal e rheumatoide habeva simile potentiales pro alterar le comportamento de fibroblastos in vitro. Erythrocytos exerceva pauc effecto super culturas de fibroblastos.

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