Leukocyte-Connective Tissue Cell Interaction. II. The Specificity, Duration, and Mechanism of Interaction Effects

By C. WILLIAM CASTOR, M.D., AND MICHAEL YARON, M.D.

Human fibroblasts grown in vitro as monolayer cultures were "activated" by syngeneic lymphocytes, polymorphonuclear leukocytes, and fibroblasts as well as by allogeneic leukocytes, human embryonic kidney cells, fibroblasts, and thrombocytes. "Activation" was characterized by increased medium acidity, increased glucose uptake, increased lactate formation, and marked stimulation of hyaluronic acid formation. The "activation" process was blocked by preincubation with actinomycin D, but not by incubation with 2,4 dinitrophenol. "Activated" fibroblasts continued to exhibit increased mucopolysaccharide synthesis for 5 to 28 days following a single exposure to stimulatory materials.

ALLOGENEIC LEUKOCYTES, in a sense, have the capacity to activate human connective tissue cells in vitro, causing increased glucose utilization and markedly stimulating hyaluronate formation.[1-3] While these experiments mimic some of the circumstances found in human homografts and thus may explain some aspects of transplant rejection phenomena, it is by no means clear that immunological mechanisms need be invoked to explain the data. In extending these observations, it seemed pertinent to examine the capacity of syngeneic leukocytes, nucleated nonleukocytes, and inert crystalline substances to induce the kind of hyperfunction of connective tissue cells described above. If it were shown that an individual's connective tissue cells could be stimulated by his own leukocytes, then these observations would have clear relevance to the proliferative (reparative) phase of inflammation. By the same token,

a more detailed examination of possible late consequences of leukocyte-fibroblast interaction and a search for the active component of stimulatory leukocytes would be warranted.

This report provides data which show that not only syngeneic leukocytes but also allogeneic kidney cells, allogeneic fibroblasts, syngeneic fibroblasts, and thrombocytes can stimulate fibroblast carbohydrate metabolism, and that the stimulatory effects linger beyond the time of the initial exposure to such "activator" materials. Studies of the mechanisms underlying "interaction" phenomena suggest that: (1) DNA-mediated synthesis of messenger RNA in the "activated" fibroblast is essential to the realization of "interaction" phenomena, and (2) the major energy surge from increased oxidation of exogenous glucose is not a prerequisite for increased hyaluronate synthesis.

C. WILLIAM CASTOR, M.D.: Professor of Internal Medicine, The University of Michigan Medical Center, Ann Arbor, Michigan. MICHAEL YARON, M.D.: Teaching Associate in Internal Medicine and Trainee in Rheumatology, The University of Michigan Medical Center, Ann Arbor, Michigan (on leave from the Beilinson Hospital, Israel).

Reprint requests should be addressed to Dr. Castor at The University of Michigan, Rackham Arthritis Research Unit, University Hospital, Ann Arbor, Michigan 48104.

Table 1.—Effect of Syngeneic Leukocytes on Synovial Connective Tissue Cells

Fibroblast strain	Diagnosis	Cell and serum additions	Final fibroblast count × 10⁶	Glucose uptake, mg./10⁶ cells/day	Total HA/flask, μg.*	HA synthesis rates, pg./cell/day
E.F.–D	RA	None, E.F. sera	3.835 (3.626–4.155)	0.29 (0.26–0.32)	226.7 (218–232)	29.7 (26.2–32.0)
		L_{EF}, E.F. sera	4.947 (4.461–5.475)	0.48 (0.33–0.56)	419.3 (408–436)	42.6 (39.8–46.4)
		PMN_{EF}, E.F. sera	4.656 (4.374–5.200)	0.52 (0.51–0.53)	384.0 (372–396)	41.4 (36.8–45.0)
		L_N, pool sera	4.899 (4.825–4.983)	0.53 (0.51–0.55)	398.7 (372–432)	40.7 (38.1–43.4)
		None, pool sera	4.108 (4.030–4.179)	0.40 (0.39–0.42)	250.0 (228–262)	30.5 (27.7–32.3)
R.L.–F	RA	None, pool sera	4.870 (4.546–5.225)	1.03 (0.99–1.10)	181.0 (152–216)	37.2 (33.4–44.6)
		L_{RL}, pool sera	5.650 (5.525–5.900)	1.39 (1.36–1.45)	389 (336–432)	69.0 (56.9–77.8)
		PMN_{RL}, pool sera	5.783 (5.300–6.250)	0.97 (0.90–1.02)	223 (207–232)	38.6 (37.1–39.7)

Fibroblast inocula were: E.F.–D = 0.992 × 10⁶. All blood cell additives were frozen and thawed three times before adding to the fibroblast monolayers. The various blood cell inocula were: L_{EF} = 5.0 × 10⁶, PMN_{EF} = 5.0 × 10⁶, L_N = 5.0 × 10⁶, L_{RL} = 2.5 × 10⁶/flask, and PMN_{RL} = 2.5 × 10⁶/flask. In experiment E.F., the interaction time was 2 days, and in R.L. it was 1 day (see text).

* In this and following tables, total HA, μg./flask, is that amount measured in the final medium change removed from the culture at the time of culture harvest and enumeration.

MATERIALS AND METHODS

Preparation, management, enumeration, and sizing of synovial connective tissue cell cultures were carried out as in earlier studies.[3-6] Medium glucose was determined by a glucose oxidase method,[7] and medium hyaluronic acid by measurement of uronic acid with a carbazole reagent after isolation of the polymer.[8] Leukocytes were prepared as noted earlier.[3] Crystalline uric acid was prepared by the method of Buchanan et al.[9] Lactic acid was measured by a colorimetric method.[10]

In general, serum dilution bottles were planted with 1.0 × 10⁶ fibroblasts in 10 ml. of medium (10 per cent human serum, 10 per cent fetal calf serum, 80 per cent synthetic medium 1066) and incubated at 37°C with complete medium changes at 2 or 3 day intervals. Foreign materials (live or dead cells, urate, etc.) were added to the fibroblast monolayers on the fifth or sixth day of the growth cycle. Details of individual experimental protocols are best visualized in connection with the descriptive text and tables. The mean values in the tables are derived from three or four flasks, and the absolute range of values found in individual flasks are recorded in parentheses.

RESULTS

Syngeneic Leukocyte-Fibroblast Interactions

When rheumatoid fibroblast monolayer cultures (E.F.-D)* were exposed to frozen-thawed leukocytes from the same patient, marked stimulation of hyaluronic acid synthesis was noted (Table 1). It is pertinent to note that the serum component of the nutrient medium also came from patient E.F. Polymorphonuclear leukocytes and lymphocytes elicited responses of similar dimensions. Cell growth was modestly increased by addition of leukocyte material, and glucose disappearance was accelerated. Lymphocytes from an allogeneic donor, in media containing an allogeneic human serum, induced increased growth and hyalu-

*The culture designation E.F.-D refers to the fourth (D) subculture of fibroblasts from a patient with initials E.F.

Table 2.—Effect of Nonleukocyte Additives on Synovial Connective Tissue Cells

Fibroblast strain	Diagnosis	Additives	Final fibroblast count × 10⁶	Glucose uptake, mg./10⁶ cells/day	Total HA/flask, μg.	HA synthesis rates, pg./cell/day
Wa.K.–G	Normal	None	7.050 (6.800–7.425)	0.21 (0.18–0.23)	211.0 (196–224)	15.0 (14.2–15.6)
Wa.K.–G	Normal	1.0 mg. uric acid/ml.	6.300 (6.075–6.475)	0.26 (0.24–0.28)	233.0 (212–248)	18.6 (16.4–20.4)
Wa.K.–G	Normal	L_N	6.697 (6.500–7.375)	0.27 (0.22–0.30)	284.0 (256–302)	20.5 (17.4–23.3)
Wa.K.–G	Normal	Hm kidney cells	4.782 (4.475–5.225)	0.73 (0.64–0.78)	290.0 (216–352)	30.5 (23.2–39.3)
Wa.K.–C	Normal	None	5.052 (4.632–5.300)	0.05 (0.03–0.09)	123.3 (114–132)	12.3 (10.9–14.2)
Wa.K.–C	Normal	Fibroblasts (C.V.)*	4.302 (4.284–4.316)	0.57 (0.56–0.58)	654.7 (636–688)	76.1 (74.1–79.9)
K.P.–G	Rheum. spond.†	None	3.31 (3.24–3.44)	0.32 (0.26–0.38)	70.7 (62–76)	10.7 (9–11.7)
K.P.–G	Rheum. spond.	0.2 mg. uric acid/ml.	3.53 (3.36–3.63)	0.32 (0.31–0.33)	65.3 (64–66)	9.3 (8.8–9.8)
K.P.–G	Rheum. spond.	1.0 mg. uric acid/ml.	3.70 (3.69–3.71)	0.32 (0.31–0.34)	89.0 (84–94)	12.1 (11.4–12.7)
K.P.–G	Rheum. spond.	L_N	3.22 (3.06–3.39)	0.99 (0.88–1.10)	138 (136–140)	21.5 (20.1–22.9)
Wa.K.–H	Normal	None	9.42 (9.12–9.60)	0.25 (0.24–0.26)	343 (340–348)	18.2 (17.8–18.6)
Wa.K.–H	Normal	Thrombocytes‡	10.30 (10.20–10.37)	0.41 (0.41–0.41)	788 (788–788)	37.8 (37.5–38.1)

* 2.5 × 10⁶ frozen-thawed fibroblasts from synovial strain C.V., derived from a 93-year-old woman, were added to each of three Wa.K. monolayer cultures.

† This culture (K.P.) was derived from the knee of an 18-year-old male with early rheumatoid spondylitis. The mean values for the various experimental groups in each case were derived from three or four flasks, except for K.P.–G treated with L_N and with 0.2 mg. uric acid/ml. where two flasks were used for each group.

‡ Normal thrombocytes added to each flask were the yield from 7.5 ml. of blood. Virtually no lymphocytes contaminated the preparation.

ronate synthesis which resembled the findings in the syngeneic combinations. No change in mean cell volume was detected.

A second rheumatoid fibroblast strain from patient R.L., showed a marked increase in HA synthesis rate and disappearance of medium glucose when exposed to syngeneic lymphocytes, but little response to syngeneic PMN leukocytes was noted. Both varieties of leukocytes caused modest stimulation of growth of strain R.L. Mean cell volume showed little change as a consequence of adding dead leukocytes. A third rheumatoid cell strain, from patient P.G. (not shown in Table 1), was minimally stimulated by frozen-thawed syngeneic lymphocytes and polymorphonuclear leukocytes. The total hyaluronate synthesis was increased by 18–56 per cent, and the specific HA synthesis rate was increased by 24–70 per cent. In this experiment, allogeneic lymphocytes and syngeneic PMN leukocytes produced the most marked increases in HA synthesis, syngeneic lymphocytes being least active in this respect.

Effects of Nonleukocyte Particulate Additives

Frozen-thawed cells of a human embryonic kidney strain,* normal allogeneic lymphocytes, and crystalline sodium urate were tested simultaneously on a normal cell strain (Table 2). All three additives interfered with culture growth, but it was clear that frozen-thawed kidney cells were at least as potent as frozen-thawed lymphocytes in accelerating glucose uptake and hyaluronate synthesis. Examination of the cultures with polarized light microscopy showed both massive aggregates and myriads of needle-like crystals lying on the cell sheet. A fibroblast strain from a patient with rheumatoid spondylitis (K.P.-G) also failed to exhibit increased hyaluronate synthesis with added uric acid, but responded as expected to normal lymphocytes (L_N). (See Table 2.) No major alteration in mean cell volume was noted with any of the additives. In another experiment (Wa.K.-C, Table 2), it was shown that allogeneic frozen-thawed human fibroblasts are also capable of inducing profound interaction effects resembling those caused by leukocytes. In another experiment (Table 6), frozen-thawed *syngeneic* fibroblasts induced a profound interaction effect in sister cultures of identical genetic make-up and culture history. Three separate experiments (one detailed in Table 2) showed that viable human thrombocytes spun from buffy coat also possess significant "activator" capability. These data would indicate that the interaction effects noted with leukocytes are not related to characteristics unique to nucleated circulating blood cells. The failure of large quantities of uric acid to duplicate the effects of broken nucleated cells suggests that some property of the cell preparations other than their particulate character was involved in eliciting enhanced HA synthesis from the fibroblast monolayer.

Post "Interaction" Behavior of Fibroblasts

When it was clear that the immediate biochemical consequences of interaction between allogeneic and syngeneic leukocytes and connective tissue cells were qualitatively similar, "follow-up" experiments were initiated to search for late consequences of a single brief interaction. Control and "interacted" fibroblast cultures were harvested, divided, and subcultured to initiate another growth cycle. It soon became obvious that second generation cultures derived from cells with prior exposure to allogeneic lymphocytes continued to produce hyaluronic acid at an accelerated pace (Fig. 1). Normal fibroblasts (strain Wa.K.) formed increased amounts of hyaluronate for at least 7 days following cessation of gross contact with leukocytes or their components. The stimulatory effect faded towards the end of the 7 day growth cycle, especially in the case of those cells exposed to nonviable lymphocytes. When these latter cultures were harvested and counted at the end of 7 days, the HA synthesis rate, calculated from the third medium change and cell count, had actually returned to control values. Cultures whose progenitors had been exposed to live allogeneic lymphocytes still exhibited an increase in HA synthesis more than 30 per cent greater than controls. Cultures which had been exposed to lymphocytes proliferated more rapidly, the final cell count being approximately 33 per cent greater than control cultures derived from nontreated forbears.

The syngeneic interaction between rheumatoid fibroblasts R.L. and leukocytes (Table 1) was followed in a similar manner. During the two subcultures following the "interaction," flasks whose progenitors had been exposed to lymphocytes continued to produce more hyaluronate and to exhibit a higher specific HA synthesis rate (Fig.

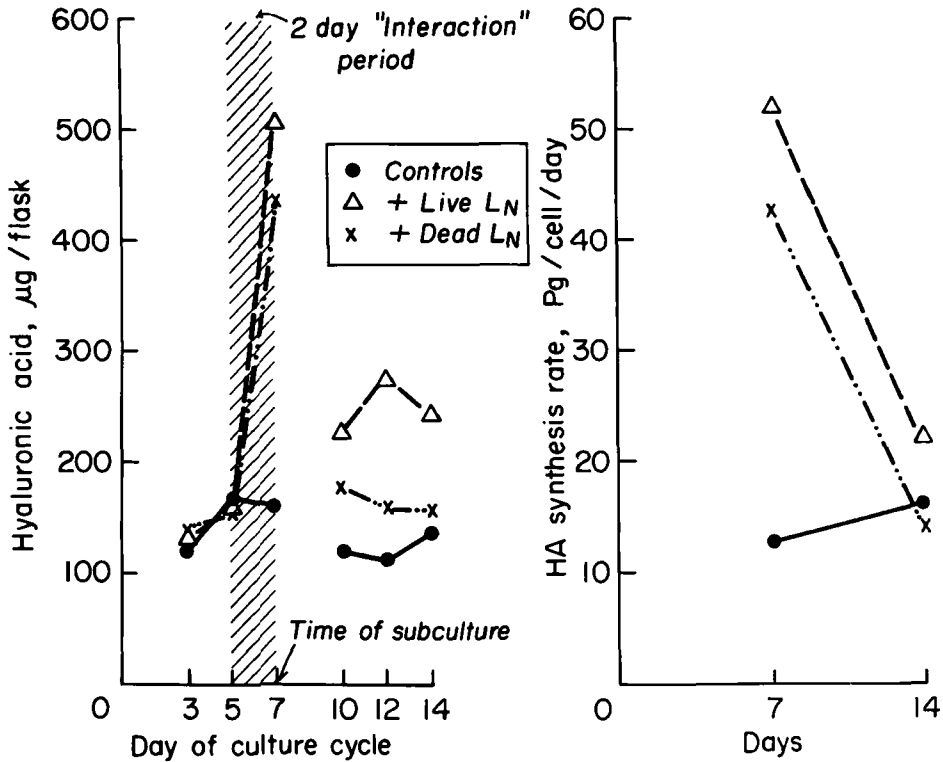*Available from Baltimore Biological Laboratory, Baltimore, Md.

Fig. 1.—Live leukocytes from a normal person (live L_N) are designated by triangles, and frozen-thawed lymphocytes from the same person (dead L_N) are represented by X's. Control flasks received no additive. The hyaluronic acid synthesis rate (right) is calculated every 7 days when the cultures are counted. The rate values presented are derived from the relation:

$$\text{Rate} = \frac{\mu g. \ \text{HA/flask}}{\text{Cell count} \times \text{duration of last medium change (days)}}$$

Hyaluronic acid/flask measures the amount formed per individual medium change.

2). Interestingly, cells derived from PMN-treated cultures, where little interaction effect had been measured initially, were clearly producing more HA than control cultures some 14 days after exposure to the leukocyte material. Figure 2 shows that hyaluronate synthesis was increased by lymphocytes during an initial 24 hour "interaction" period, but the most striking increment occurred another 24 hours later, suggesting that time-dependent modifications of cellular synthetic systems were required. During the 1 day interaction, when medium glucose was not entirely depleted,

actual stimulation of cell growth was observed, leukocyte-treated cultures showing final cell counts 19–24 per cent higher than controls.

To further explore the duration of the induced alterations, the syngeneic experiment with strain E.F. (Table 1) was observed through four subcultures (28 days) following exposure to leukocyte material (see Fig. 3). It is clear from Fig. 3 that growth stimulation was most marked where lymphocytes had been added and that this effect died out with subsequent subcultures. On the other hand, total amount of hyalu-
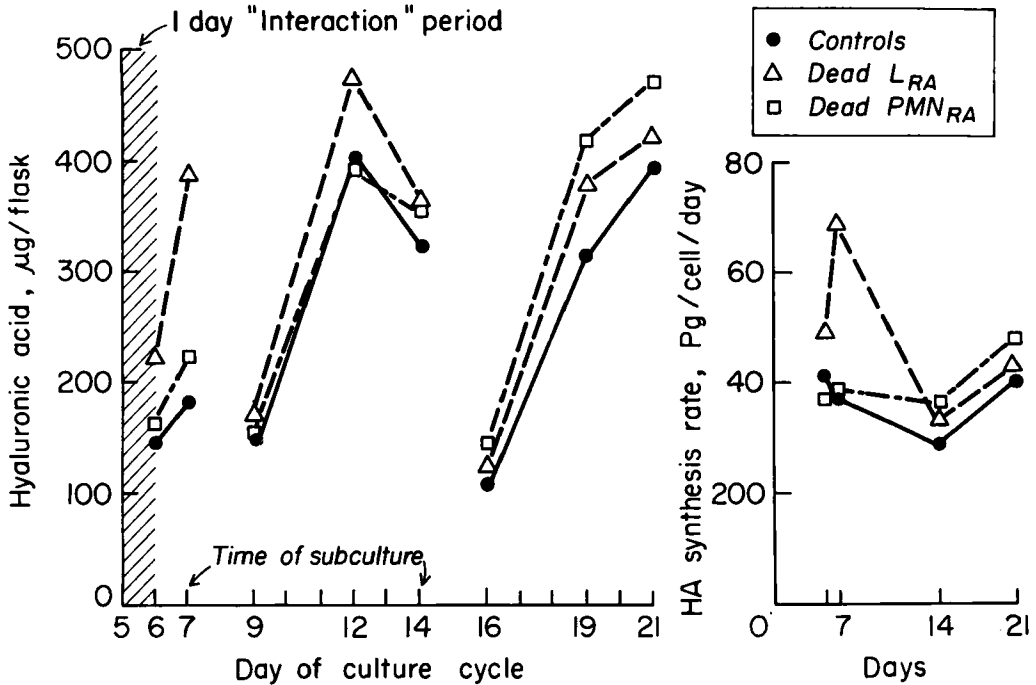
Fig. 2.—Frozen-thawed lymphocytes (dead L_{RA}) and polymorphonuclear leukocytes (dead PMN_{RA}) were harvested from rheumatoid patient R.L. and interacted with fibroblasts grown from R.L.'s synovial membrane. Hyaluronic acid measurements are the amounts formed in a single medium change.

ronate formed and the specific hyaluronate synthesis rate remained higher than control values for the entire follow-up period.

Studies on the Mechanism of "Interaction" Effects

From the data, it appeared that some constituent(s) in several varieties of human cell caused living human fibroblasts in monolayer culture to rapidly consume glucose, acidify the medium, markedly stimulate hyaluronic acid formation, and modestly stimulate (or retard) growth. The immunologic relatedness of the interacting cells appeared to be of little consequence. The health of the donors of fibroblasts and other cells did not play a controlling role, and it was not even necessary that the cells added to the fibroblast monolayers be alive.

Although accelerated glucose disappear-

ance from culture medium always accompanied the increased hyaluronate synthesis when fibroblasts were "activated," it is not certain that these two findings are necessarily directly related. On the other hand, it seemed reasonable to suppose that the energy from increased glucose oxidation might be required for increased macromolecule synthesis. To determine whether the large amount of energy available from increased glucose oxidation was essential for increased hyaluronate synthesis, we exposed appropriate cultures to 2.5×10^{-4} M 2,4-dinitrophenol, as shown in Table 3. That this inhibitor of oxidative phosphorylation was effective is suggested by the profound increase in glucose consumption caused by the drug. Addition of frozen-thawed connective tissue cells to the fibroblast monolayer induced the expected increment in
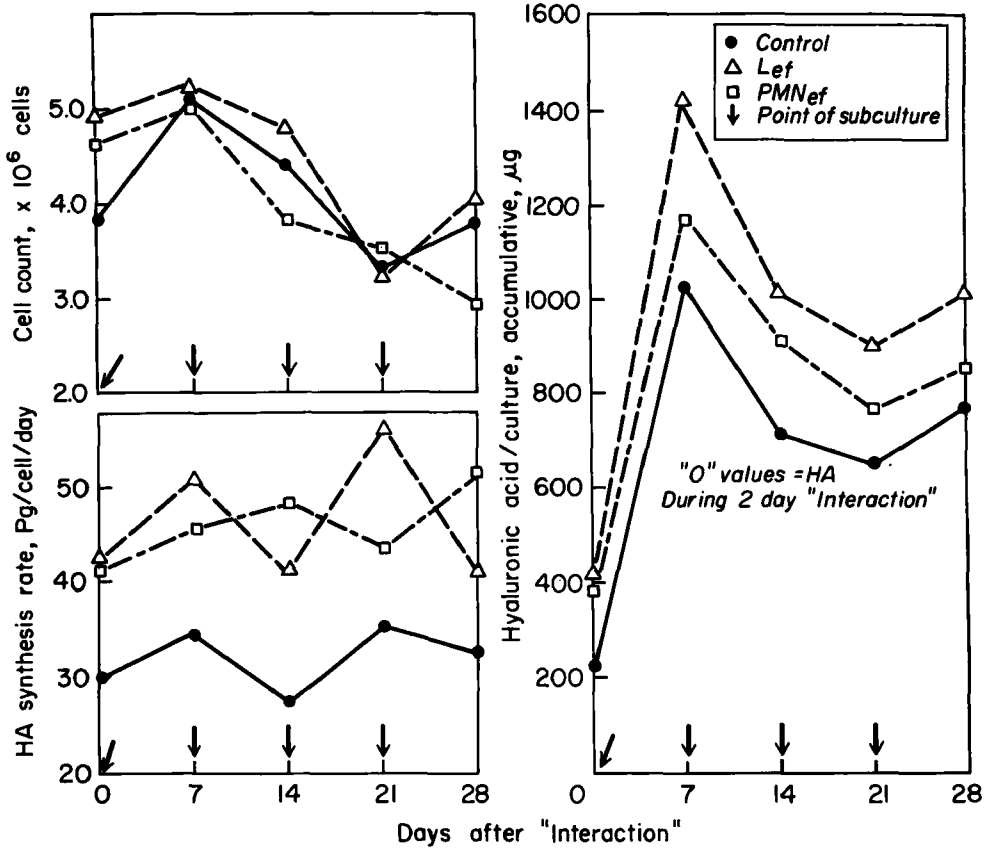
Fig. 3.—The accumulated hyaluronic acid values plotted in the right panel at 7, 14, 21, and 28 days record the total HA/flask in the preceding week, that is, the sum of the three individual medium changes for each culture. Each point reflects the mean of values derived individually from three flasks.

HA synthesis, and this increased rate of mucopolysaccharide synthesis was not blocked in any major sense by 2,4-dinitrophenol. It is pertinent to note that the specific rate of HA synthesis per cell was virtually unchanged by the inhibitor, the decrease in total HA formed being largely explained by the decreased number of cells. The data indicate that the energy derived from phosphorylation at the substrate level, hence insensitive to dinitrophenol, is adequate to meet the demands of increased hyaluronate synthesis. Preservation of extracellular macromolecular synthesis in the face of wastage of 85 per cent of the energy

available from exogenous glucose (i.e., energy associated with oxidative phosphorylation) suggests that this cell function has high priority when energy resources are restricted. In view of these findings, it seems likely that, while some of the extra energy realized from accelerated glucose utilization serves to support increased hyaluronate synthesis, the major portion must be expended in other ways.

Although increased lactic acid formation is clearly a consequence of fibroblast "activation," it does not account for all of the additional glucose removed from the medium as a result of this process. (See Table

Table 3.—Failure of 2,4-Dinitrophenol to Block Fibroblast "Activation"

Fibroblast strain	Diagnosis	Additives	Final fibroblast count × 10⁶	Glucose uptake, mg./10⁶ cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
B.S.–F	Osteoarthritis	None	8.656 (8.325–8.925)	0.07 (0.02–0.16)	109 (92–126)	6.3 (5.5–7.4)
B.S.–F	Osteoarthritis	2,4–DNP *	8.113 (7.550–8.500)	0.49 (0.47–0.54)	97.0 (72–140)	6.0 (4.4–8.2)
B.S.–F	Osteoarthritis	Fibroblasts,† Wa.K., frozen-thawed	9.569 (9.000–10.450)	0.14 (0.08–0.18)	280.5 (272–288)	14.7 (13.5–15.9)
B.S.–F	Osteoarthritis	2,4–DNP + fibroblasts	7.394 (7.250–7.525)	0.55 (0.54–0.56)	187.3 (175–200)	12.7 (11.9–13.5)

* 2,4-Dinitrophenol was added to a final medium concentration of 2.5×10^{-4} M.

† The frozen-thawed Wa.K. fibroblasts were from a "normal" strain. Approximately 3.15×10^6 were added to each experimental flask.

Table 4.—Increased Lactate Formation with "Activation"

Fibroblast strain	Diagnosis	Additives	Final fibroblast count × 10⁶	Glucose uptake mg./10⁶ cells/day	Lactate output, mg./10⁶ cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
Wa.K.–N	Normal	None	5.417 (5.275–5.525)	0.20 (0.20–0.21)	0.09 (0.08–0.09)	224 (220–228)	13.8 (13.3–14.4)
Wa.K.–N	Normal	R.L. extract*	4.228 (3.992–4.398)	0.62 (0.60–0.66)	0.35 (0.34–0.36)	1091 (992–1152)	86.2 (77.0–94.2)
Wa.K.–L	Normal	None	4.851 (4.722–4.988)	0.48 (0.48–0.48)	0.30 (0.30–0.30)	381 (368–400)	39.2 (38.2–40.5)
Wa.K.–L	Normal	E.F. extract†	4.175 (3.993–4.418)	0.87 (0.86–0.87)	0.61 (0.57–0.65)	671 (632–708)	80.3 (79.1–81.7)

* A saline extract of rheumatoid fibroblast strain R.L. was added in an amount equivalent to 2.8×10^6 cells per flask.

† E.F. extract was a saline extract of rheumatoid fibroblast strain from patient E.F. added in an amount equivalent to 3.0×10^6 cells per flask.

4.) To assess the role of excess lactic acid, cultures were subjected to low pH in one case and excess lactate (neutralized) was added to another group. As shown in Table 5, excess hydrogen ion had an adverse effect on both cell growth and total HA synthesis, but virtually no effect on the specific rate of hyaluronate formation. Lactate, however, appeared to modestly increase both total hyaluronate synthesis and the specific synthesis rate, although not to the same degree seen in the majority of the interaction experiments. In a second normal cell strain (C.V.-E), the magnitude of the lactate-induced stimulation of hyaluronate synthesis was related to the total amount of lactate added to the culture medium (Table 5). In experiments not presented in detail here, it appears that pyruvate partially mimics lactate, and that 2,4-dinitrophenol does not block the stimulatory effect of either of these substances on hyaluronate synthesis. While it seems likely that the increased amount of lactate from accelerated glycolysis may account for part of the increased HA synthesis following fibroblast "activation," the precise mechanism is as yet uncertain.

In view of an apparent time lag between addition of "activating" substances and the observed effects, the possibility that gene activation might be required was explored. Since previous experiments had shown human fibroblasts in vitro to be very sensitive (growth inhibition) to actinomycin D, we pretreated the fibroblast cultures for 6

Table 5.—Effects of Excess Lactate and Hydrogen Ion

Fibroblast strain	Diagnosis	Additives	Final fibroblast count × 10⁶	Glucose uptake, mg./10⁶ cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
Wa.K.–D	Normal	None	5.193 (4.802–5.650)	0.29 (0.26–0.32)	168 (160–180)	16.2 (14.5–18.7)
Wa.K.–D	Normal	Lactate, 1.0 mg./ml.	5.438 (5.225–5.625)	0.22 (0.17–0.24)	222 (212–236)	20.5 (18.8–22.4)
Wa.K.–D	Normal	Hydrogen* ion	3.816 (3.529–4.043)	0.27 (0.26–0.28)	106 (104–110)	13.9 (13.2–14.7)
C.V.–E	Normal	None	4.314 (4.107–4.472)	0.49 (0.48–0.51)	456 (448–462)	35.3 (33.4–37.2)
C.V.–E	Normal	Lactate, 1.0 mg./ml.	4.717 (4.652–4.768)	0.44 (0.43–0.45)	553 (548–558)	39.3 (38.6–40.0)
C.V.–E	Normal	Lactate, 2.0 mg./ml.	4.230 (3.897–4.706)	0.45 (0.42–0.48)	645 (628–672)	51.2 (44.5–54.8)

* Cultures were rendered acid by omitting usual addition of Na_2CO_3, thus producing pH of approximately 7.0 (judged by phenol red indicator in media) as opposed to pH 7.4–7.6 under normal conditions.

Table 6.—Actinomycin D Effects on Fibroblast "Activation"

Fibroblast strain	Diagnosis	Additives	Final fibroblast count × 10⁶	Glucose uptake, mg./10⁶ cells/day	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
K.P.–F	Rheum. spond.	None	6.450 (6.226–6.775)	0.32 (0.30–0.34)	116.0 (102–124)	9.0 (8–10)
K.P.–F	Rheum. spond.	Actinomycin D, 0.1 μg./ml.	4.260 (4.001–4.655)	0.56 (0.52–0.59)	80.7 (68–88)	9.5 (8.5–10.4)
K.P.–F	Rheum. spond.	Fibroblasts,* Wa.K., frozen-thawed	8.025 (7.875–8.200)	0.39 (0.38–0.40)	213.3 (208–216)	13.3 (13.2–13.5)
K.P.–F	Rheum. spond.	Actinomycin D + fibroblasts	4.363 (4.221–4.457)	0.52 (0.50–0.53)	72.0 (68–74)	8.3 (8.1–8.4)
Wa.K.–G	Normal	None	6.894 (6.800–7.025)	0.33 (0.30–0.35)	330.5 (328–336)	24.0 (23.4–24.4)
Wa.K.–G	Normal	Actinomycin D, 0.1 μg./ml.	3.880 (3.689–4.078)	0.60 (0.57–0.62)	326.5 (306–344)	42.1 (40.2–44.5)
Wa.K.–G	Normal	Dead fibroblasts,* Wa.K.–G	5.556 (5.250–5.850)	0.77 (0.73–0.81)	834.0 (800–848)	75.2 (70.2–80.4)
Wa.K.–G	Normal	Dead fibroblasts + actinomycin D	4.120 (3.975–4.303)	0.61 (0.55–0.65)	357.5 (336–376)	43.4 (40.6–45.3)

* In the K.P. experiment, 3.34 × 10⁶ dead Wa.K. cells were added per flask, and in the Wa.K.–G experiment, 3.1 × 10⁶ dead syngeneic Wa.K.–G cells were added per flask.

hours with 0.1 μg. actinomycin D/ml. of medium before adding the "activating" lymphocytes. During the 48 hour "interaction" period the actinomycin D was left in contact with the cultures. In an experiment with fibroblast strain K.P.-F (Table 6), complete blockade of fibroblast "activation" was achieved. Of interest is the fact that the antibiotic blocked *both* increased glucose consumption and increased hyaluronate synthesis by fibroblast cultures exposed to an effective "activator." In the syngeneic interaction between frozen-thawed Wa.K. cells and the same strain in monolayer culture, typical "activation" occurred and was blocked by 0.1 μg. actinomycin D/ml. of medium (Table 6). The apparent stimulatory effect of actinomycin

Table 7.—Presence of Activator Substance(s) in Medium from Lymphocyte Cultures

Fibroblast strain	Diagnosis	Additives	Final fibroblast count × 10⁶	Total HA/flask, μg.	HA synthesis rate, pg./cell/day
B.S.–I	Osteoarthritis	None	2.170 (1.98–2.30)	61.3 (60–62)	28.4 (27.0–31.3)
B.S.–I	Osteoarthritis	L_N, freeze-thawed	2.85 (2.39–3.64)	97.3 (88–108)	35.7 (24.2–42.7)
B.S.–I	Osteoarthritis	Live L_N, incubated	2.17 (2.09–2.35)	65.3 (58–76)	30.1 (27.4–32.3)
B.S.–I	Osteoarthritis	Media from incubated L_N	2.28 (2.25–2.32)	100.7 (88–116)	44.1 (39.1–50.0)

D alone on the hyaluronate synthesis rate may merely reflect failure of the culture to divide in the presence of the antibiotic. It should be noted that "activation" following actinomycin D gives the same final cell count, glucose uptake rate, total HA, and HA synthesis rate as actinomycin D alone, values quite unlike those found in the unblocked interaction. These experiments provide evidence that nuclear participation by the "activated" fibroblasts is required for "interaction" effects, presumably in the form of synthesis of DNA-dependent ribonucleic acid.

In experiments with two different fibroblast strains, it was possible to demonstrate "activator" substance in medium in which lymphocytes had been incubated for 3 days prior to being added to fibroblast monolayers. In Table 7, it is clear that media from the lymphocytes which had been incubated in vitro had a stimulatory influence on hyaluronate synthesis, while the cells themselves were without significant influence.

The composition of the materials(s) found in lymphocytes, polymorphonuclear leukocytes, human embryonic kidney cells, and cultured connective tissue cells which induces "activation" of monolayer fibroblast cultures is at present under study. Our early studies demonstrated that frozen-thawed lymphocyte preparations, when fresh, were equally as potent as live cells, but tended to lose potency on prolonged storage in the frozen state. In addition, polymorphonuclear leukocytes lose their "activator" substance(s) rather quickly after freeze-thawing. It seemed possible that this lability might be attributed to enzymatic destruction of the activator substance following breakdown of cell structure by freeze-thawing.

The proteolytic enzyme pronase partially destroyed thrombocyte "activators" in one experiment, and damaged the activator activity of frozen-thawed fibroblasts in other experiments, suggesting that activator materials are protein in nature. Repeated attempts to "activate" fibroblasts with lysosomes isolated from human leukocytes have not produced clear changes in fibroblast behavior.

DISCUSSION

The inflammatory process resulting from simple tissue injury is initiated by an acute vascular phase (congestion, stasis, increased vessel permeability), then progresses rapidly to an exudative phase, (edema, fibrin formation, leukocyte emigration, and phagocytosis), and finally culminates in a reparative stage characterized by fibroblast proliferation, elaboration of ground substance mucopolysaccharide, and finally collagen deposition. Samples of chronically inflamed synovium from patients with rheumatoid arthritis usually exhibit all phases of the inflammatory process in varying proportions. Microscopically, one sees

proliferation of synovial lining cells and stromal connective tissue cells and areas of collagen deposition (fibrosis). The adjacent joint cavity, a specialized connective tissue space, contains excessive quantities of underpolymerized hyaluronic acid produced by the synovial tissue.[11] Increased mucopolysaccharide synthesis during inflammation is not peculiar to synovial membrane, but has been documented in the polyvinyl sponge granuloma,[12] and in tissue exhibiting the Shwartzman phenomenon.[13] The known metabolic abnormalities of rheumatoid synovial inflammation include not only marked overproduction of hyaluronic acid but also greatly accelerated glucose utilization, increased glycolysis, and oxidative metabolism.[14]

The present experiments simulate rheumatoid synovitis in the sense that the same cellular components were brought together, that is, synovial connective tissue cells, lymphocytes, and polymorphonuclear leukocytes. More importantly, the biochemical consequences of adding leukocytes to fibroblast cultures mimic synovitis with respect to accelerated glucose utilization and to increased hyaluronate synthesis. Viewed in this light, these "interaction" experiments suggest that leukocytes, and indeed many other cell types, bear "activator" substances capable of causing the fibroblast to initiate some of the synthetic activities characteristic of the reparative phase of inflammation.

"Activation" of fibroblasts in culture by leukocytes from the fibroblast donor has special significance, since it indicates that histocompatibility is no barrier to the activation phenomenon. A corollary observation would suggest that antigenic heterogeneity among the reactants is not required to initiate fibroblast "activation." This interpretation of the syngeneic experiments presupposes that no significant antigenic alteration of the cultivated fibroblasts occurs during the time required to establish a monolayer culture. There is evidence that

certain species-specific antigens remain as stable cell characteristics during in vitro culture,[15] but it is also clear that primary cell strains may both gain and lose antigens during the culture process.[16] In short, the syngeneic combination of leukocytes and fibroblasts may be less than truly syngeneic if significant fibroblast antigen alteration occurred during the period of culture development. These theoretical considerations seem less important, however, in view of the potent "activation" of living fibroblasts by frozen-thawed brethren of the same strain and passage status.

Some important characteristics of "activator" substances have been defined by the current experiments. Negative results with uric acid, erythrocytes, and aged PMN extracts suggest that activation requires more than a nonspecific particulate stimulus. The presence of "activators" in media from lymphocyte cultures indicates that these materials are soluble substances secreted by, or leached out of, viable lymphocytes.

Actinomycin D blockade of the activation process suggests that DNA-mediated synthesis of RNA is necessary to bring about hyperfunction of connective tissue cells. This feature in itself may distinguish the "activation" phenomenon described in this report from allogeneic cytotoxicity, since the cytotoxic effect of human lymphocytes on Chang liver cells is apparently not blocked by actinomycin D, 10 μg./ml.[17]

Cellular materials which activated fibroblasts with respect to glucose and hyaluronate metabolism usually altered the growth performance of target fibroblasts as well. In 16 of 21 experiments the final cell count in the "activated" fibroblast population was modestly depressed (about 20 per cent) below control flasks. However, in five experiments, clear stimulation of growth by about the same amount was observed. We have no explanation for these divergent growth responses, although the findings may be analogous to those of Ryan

and Cardin.[18] These investigators found evidence for nondialyzable, thermolabile protein(s) in liver lysosomes which stimulated growth of strain L fibroblasts in low concentrations and inhibited growth in high concentrations. It is not clear at present whether the genesis of the growth changes lies with the activator(s) of carbohydrate metabolism or in an entirely separate mechanism.

One of the interesting aspects of the activation process which may be important in the pathogenesis of inflammatory disease is the lingering nature of the increase in hyaluronate synthesis after a single short exposure to leukocyte materials. It may be that dilution and biological degradation of activator substance(s) subject the fibroblast population to a continued but diminishing stimulus. *The activator material(s) described here may play a central role in initiating and regulating the progression of the inflammatory process from the exudative to the reparative (proliferative) phase.*

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SUMMARIO IN INTERLINGUA

Fibroblastos human, crescite in vitro como culturas monostratal esseva "activate" per syngenic lymphocytos, leucocytos polymorphonucleari, e fibroblastos e etiam per allogenic leucocytos, embryonic cellulas renal human, fibroblastos, e thrombocytos. Le "activation" esseva characterisate per un augmentate aciditate del medio, augmentos del acceptation de glucosa, augmentos del formation de lactato, e marcate stimulation del formation de acido hyaluronic. Le processo del "activation" esseva bloccate per preincubation con actinomycina D sed non per preincubation con 2,4-dinitrophenol. Fibroblastos "activate" continuava exhibir un augmentate synthese de mucopolysaccharidas durante 5 a 28 dies post un sol exposition a materiales stimulatori.

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