Effects of Rheumatoid Sera on Fibroblast Proliferation and Hyaluronic Acid Synthesis

By C. WILLIAM CASTOR, DELOISTEEN WRIGHT AND R. B. BUCKINGHAM

Rheumatoid serum depresses synovial fibroblast proliferation *in vitro* and tends to enhance the rate of hyaluronic acid formed per cellular unit. Quantitatively, the observed effects are not large, are apparently not disease specific, and not related to the titer of rheumatoid factor as measured by latex fixation. An improved method for measuring acid mucopolysaccharide in tissue culture medium is described.

THE rheumatoid inflammatory process is characterized by uncontrolled connective tissue cell proliferation, overproduction of specialized cell products, such as hyaluronic acid, colonization of affected tissues by various types of "inflammatory cells," and alterations in the microvasculature. Once established, this complex process is "self-perpetuating" and leads to destruction of capsular structures, cartilage, and bone with consequent impairment of articular function. To identify and measure the important interrelationships in the rheumatoid granuloma we have employed cell culture techniques which permit intensive study of isolated segments of the problem in a simplified system.

There is little literature dealing with the consequences of incorporating rheumatoid sera into nutrient medium to support human articular cells *in vitro*. One study concluded that the ability of synovial cells to spread on glass in tissue culture was inhibited by human serum, and that rheumatoid serum was significantly more active in this respect than was normal serum.[1] This inhibitory phenomenon was obliterated in both normal and rheumatoid sera by heat inactivation at 56 C for 30 minutes. Another report concluded that rheumatoid serum did not influence the intrinsic viscosity of hyaluronic acid synthesized by synovial tissue cultures derived from patients with rheumatoid arthritis and degenerative joint disease, but the point in the culture growth cycle when measurements were made in this study would not have been conducive to demonstrating serum-induced changes of intrinsic viscosity.[2]

Earlier studies in this laboratory lead us to suggest that connective tissue cells derived from normal and rheumatoid synovial membrane exhibited measurable differences in function, differences which were transferable from one generation of cells to the next.[3] This report deals with one element in a general study of connective tissue cell function in rheumatic dis-

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This investigation was supported by USPHS Grant AM 10728.

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Table 1.—*Characteristics of Rheumatoid Sera Donors*

Donor	Rheumatoid factor (reciprocal titer)	Aspirin tablets/day	Other drugs/day
1. L.B.	1280	8	200 mg. Hydroxychloroquin
2. S.W.	5120	8	400 mg. Hydroxychloroquin
3. N.V.	5120	12	Colchicine (3)
4. A.R.	5120	12	Gold
5. F.D.	5120	16	Gold
6. G.S.	5120	16	Gold
7. A.H.	5120	16	Isordil
8. V.B.	5120	17	Digitoxin 400 mg. Hydroxychloroquin
9. R.W.	2560	24	Gold, Indocin
10. T.M.	0	12	Gold

eases, that is: the regulatory influence of humoral (serum) factors on synovial connective tissue cells of rheumatoid and nonrheumatoid origin. In particular, the study is addressed to the possibility that factors in sera from patients with rheumatoid arthritis may modify functional activities of human articular connective tissue cells in ways which may be measured quantitatively *in vitro*. Cellular proliferative rate, hyaluronic acid synthesis rate and the molecular weight of culture-produced hyaluronate were measured and compared in cultures supported by normal and rheumatoid sera.

Methods

Procedures used for establishing, propagating, and subculturing cell strains from normal and pathologic synovial tissue were outlined in earlier publications.[4,5,6] Enumeration and sizing of trypsin dispersed cells were carried out with a Coulter Model B cell counter. Sera were harvested from blood samples which had clotted overnight at 6 C. Erythrocytes were removed by centrifugation at 3000 rpm, 6 C. Sterilization was accomplished by vacuum filtration through a No. 6 Seitz pad at 6 C. Heat inactivation, when indicated, was carried out at 52 C for 30 minutes. Tissue culture medium was composed of synthetic medium 1066* (80 per cent), fetal calf serum* (10 per cent), and human serum (10 per cent), supplemented with L-glutamine and 100 μg./ml. of penicillin G and streptomycin.

Hyaluronic acid (HA) in culture media was measured by a new procedure which is more accurate, more sensitive and far less time-consuming than our previous method.[7] Our current method is outlined in detail:

Alcohol precipitation. Two ml. of medium sample is transferred to a 15 ml. Corex tube and 8 ml. of absolute ethanol is added and mixed thoroughly (Vortex). Let this mixture stand for 1 hour at room temperature and mix 2 or 3 times during this interval. Centrifuge the sample for 10 minutes at 12,000 rpm (25–27 C), decant supernatant fluid and save precipitate.

Lipid extraction. Add 3 ml. of acetone to the precipitate, mix vigorously (Vortex) and allow to stand for 30 minutes at room temperature. Centrifuge sample as above, and decant as much supernatant fluid as possible without breaking up the precipitate. The solvent remaining will evaporate overnight if tubes are left uncovered.

Pronase† digestion. The protein-mucopolysaccharide precipitates should be thoroughly dry before starting proteolytic digestion. To each sample add 2 ml. of pronase solution containing 1.0 mg. Pronase/2 ml. .05 M Tris buffer, pH 7.8–8.0. Incubate at 37 C for 1 hour with periodic mixing. The precipitate should be completely dissolved

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†Pronase, a proteolytic enzyme formed by Streptomyces griseus was obtained from California Corporation for Biochemical Research, Los Angeles, Calif.

Table 2.—*Effects of Rheumatoid Sera on Cultured Fibroblasts*

Synovial cell strain	Human serum source	Final cell ct $\times 10^6$	MCV (μ^3)	HA/flask final medium change (μg.)	HA synthesis rate (pg/cell/day)
MM-E*	Normal	6.225	2091	276	14.7
MM-E	RA-Pool	4.859	1734	576	39.5
IP-G	Normal	4.144	2193	139	11.2
IP-G	RA-Pool	4.256	2652	516	40.4

*MM denotes the initials of the donor of the cell strain, and the "E" refers to the passage status of the strain. Thus, MM-A would represent the first trypsin subculture from the explant, while MM-E was in its fifth passage.

at the end of this period. If not, continue incubation.

Cetylpyridinum chloride precipitation. Dilute the Pronase mixture with 5 ml. double distilled water and mix thoroughly. Add 0.2 ml of 5 per cent CPC in 0.2 M Na_2SO_4 and allow to flocculate for either 1 hour at 37 C or overnight at room temperature. Collect the precipitate by centrifugation at 15,000 rpm for 10 minutes (25–27 C). This precipitate (CPC-HA complex) appears as a haze on the side of the tube and decantation of supernatant fluid should be performed carefully. Dissolve CPC-HA precipitate in 2 ml. of 2.0 M, pH 7.0 sodium acetate by warming to 37 C and mixing at intervals for 30 minutes. Use 1.0 ml. of this preparation to analyze for uronic acid by a borate carbazole procedure.[8]

Blanks consisting of unused media accompany each determination, and the optical density yielded by the blank media is subtracted from the sample absorbency before reading the corresponding μM value of the sample from the uronic acid standard curve. The μMoles of uronic acid determined represent that present in 1.0 ml. of the original media, and where the kind of mucopolysaccharide (MPS) contributing the uronic acid is known, the weight of the MPS/ml. is obtained by multiplying μM uronic acid times M.W. of

Table 3.—*Effects of Individual and Pooled Rheumatoid Sera on Cultured Human Fibroblasts*

Synovial cell strain	Human serum source	Final cell ct $\times 10^6$	MCV (μ^3)	HA/flask final medium change (μg.)	HA synthesis rate (pg/cell/day)	Per cent change
MM-G	Normal	4.662	2618	217	23.2	—
MM-G	RA-(NV)*	3.374	2312	192	28.4	+22
MM-G	RA-(VB)	3.985	2312	201	25.3	+ 9
MM-G	RA-(GS)	3.770	2448	217	28.8	+24
MM-G	RA-(LB)	3.075	2584	237	38.6	+66
MM-G	RA-(AR)	3.351	2686	238	35.5	+53
IP-H	Normal	3.189	2652	132	20.7	—
IP-H	RA-(AH)	2.909	2652	167	28.9	+40
AM II-G	Normal	3.998	2143	161	20.3	—
AM II-G	RA-(Pool)†	3.659	2253	192	26.4	+30
MaB-F	Normal	4.081	2903	122	15.5	—
MaB-F	RA-(Pool)†	3.172	2923	125	19.7	+27

*Parentheses include initials of individual serum donors. Sera labeled "normal" was from the same pool (3 normal donors) in each instance. Strain MM was from a patient with traumatic synovitis, AM II was from normal synovium, while I.P. and MaB were from rheumatoid synovitis.

†The RA sera "pool" used on AM-II consisted of equal portions of sera L.B., G.S., V.B., and A.H. The RA sera pool for MaB consisted of equal portions of sera R.W. and T.M.

Table 4.—*Effects of Individual and Pooled "Normal" Sera on Cultured Human Fibroblasts*

Synovial cell strain	Human serum source	Final cell ct $\times 10^6$	MCV (μ^3)	HA/flask final medium change (μg.)	HA synthesis rate (pg/cell/day)	Per cent change
WaK-H	Pool 182-H.I.*	4.615	2300	112.7	12.2	—
WaK-H	Pool A,B,AB-H.I.†	5.072	2333	124.7	12.6	+ 3
WaK-H	Pool A,B,AB-Not H.I.	4.890	2200	132.7	13.6	+11
WaK-H	A-Not H.I.	5.026	2333	127.6	12.8	+ 5
WaK-H	B-Not H.I.	5.466	2133	122.0	11.2	− 8
WaK-H	AB-Not H.I.	5.321	2133	135.3	12.7	+ 4

*Pool 182 consisted of 3 normal donors and H.I. means "heat inactivated at 52°C, 30 min."

†The designation A,B,AB of the individual donors refers to their blood types. Values in the table are the mean of 3 flasks. Variation in values within groups was of similar magnitude to the variation between group means noted above (i.e. 10% or less).

the appropriate dissacharide. Mean recovery of HA added to culture media was 95.7 per cent ± standard deviation of 7.5 per cent in 71 consecutive trials. Good recoveries were obtained over the range of 1.0 to 100 μg./ml. of medium. Comparison of media samples analyzed by the previous (dialysis) procedure[7] and by the procedure outlined here show good agreement. Viscometric measurement of hyaluronate molecular weight was performed with Ostwald viscometers, as described earlier.[7]

Media from the cell cultures were tested for rheumatoid factor or rheumatoid factor-like activity by the multiple tube dilution technique described by Singer and Plotz.[9] End point titers were done beginning with the whole media which contained human serum in a dilution of 1:10. Agglutination was read with the unaided eye immediately after incubation and centrifugation. None was refrigerated before reading.

The pH of the diluted media, following addition of the R.A. buffer, latex particles and plasma fraction II, was determined to ensure that agglutination was not the result of variations in pH.* No significant variations from the pH of the RA buffer reagent were found.

Cells from cultures were washed once with physiologic saline, centrifuged at 800 rpm and resuspended in one ml. of 0.9 per cent saline solution. This suspension was then exposed to ultrasonic vibration at 3 amp intensity for one minute using a Branson Sonifier.[10] A portion of the sonicated suspension was clarified by centrifugation for 10 minutes at 2300 rpm in a table top centrifuge. End point titers were performed by the method of Singer and Plotz, on the gross cell sonicates, and on the clear supernates obtained after centrifugation. Testing began at a dilution of 1:2 with careful monitoring of pH. Donors of rheumatoid sera were taking a variety of drugs and in most cases had a positive latex fixation test for rheumatoid factor (Table 1).

*Reagents used were Difco Bacto R.A. Buffer, Difco Bacto Latex, and Difco R.A. Plasma Fraction II, purchased from Difco Laboratories, Detroit, Mich.

Results

In a preliminary experiment pooled sera from 2 patients with rheumatoid arthritis (RA) were incorporated as the human serum component in media for cell strains derived from patients with traumatic synovitis (MM) and rheumatoid arthritis (I. P.). In view of the striking stimulation of hyaluronic acid elaborated by each strain (Table 2), a more detailed study was undertaken. Rheumatoid sera from individuals and from pools of 3 patients each were tested, using normal donor serum pools (3 persons) as controls. To minimize the effects of serum manipulation and avoid

Table 5.—*Effects of Individual Polycythemic Sera on Human Fibroblasts*

Synovial cell strain*	Human serum source	Final cell ct $\times 10^6$	HA/flask final medium change (μg.)	HA synthesis rate (pg/cell/day)
F_2D-F	Normal (87)	6.811	383	28.7
F_2D-F	Normal (88)	5.519	367	33.9
F_2D-F	Polycythemia (89)	2.030	103	24.8
DE-S-F	Normal (115)	3.687	198	26.9
DE-S-F	Polycythemia (104)	3.022	85	14.1
DE-S-F	Polycythemia (105)	2.482	194	39.1†
DE-S-F	Polycythemia (106)	3.476	178	25.6
EM-E	Normal (130)	3.500	290	41.3
EM-E	Polycythemia (123)	2.986	282	51.1†
EM-E	Polycythemia (127)	3.371	178	26.6

*Strain F_2D was of rheumatoid origin, DE-S and EM were of normal origin.

†Sera 105 and 123 from patients with polycythemia rubra vera altered culture performance much as did rheumatoid sera.

masking heat-labile agents, no heat inactivation step was used in serum preparation.

Both individual rheumatoid sera and pooled rheumatoid sera were less effective in supporting cell proliferation than was normal serum (Table 3). As recorded in Table 3, the defective growth-promoting capacity of rheumatoid serum was shown for two rheumatoid strains (I.P. and MaB), a "normal" strain (AM II), and a strain derived from a patient with traumatic synovitis (MM). No consistent alterations in cell volume were noted in cultures nourished by rheumatoid serum. Hyaluronate synthesis, measured on a rate basis (pg/cell/day), was increased in the presence of rheumatoid serum in all cases, the average increase in apparent synthesis rate being 39 per cent. In 4 of the 8 comparisons the increased HA synthesis rate by R.A.-sera treated cultures was largely explained by decreased cell duplication in the face of absolute amounts of HA synthesis resembling controls, but four experiments exhibited both decreased culture growth and increased total HA formation. This latter group demonstrated the largest percentage increase in HA synthesis rate induced by RA-sera (30 to 66 per cent).

A major concern in the interpretation of Table 3 revolves around the question of variability in culture response to *any serum*, whether from patients with rheumatoid arthritis, from patients with other diseases, or from normal persons. The performance of a typical normal synovial cell strain in response to 6 different human serum preparations is illustrated in Table 4. Growth, total HA synthesis and the HA synthesis rate agreed within about 10 per cent irrespective of serum preparation. This experiment suggests that neither pooling of normal sera nor heat inactivation affects the parameters measured.

Our records indicate that HA synthesis rates measured at different times in the life history of a cell strain (i.e. at different *in vitro* ages and/or different transfer ages), and using different normal sera, may vary by 4 to 60 per cent from the mean performance value* for the strain. Even a

*Mean performance value is used here to connote the arithmetic average of all measurements of a given parameter made during the *in vitro* history of a cell strain prior to obvious senescent changes.

Table 6.—*Rheumatoid Factor Titer in Culture Medium After Exposure to Fibroblasts*

Synovial cell strain	Human serum source	R.F. titer of unused medium	Rheumatoid factor titer From cultures		
			1st change	2nd change	3rd change
Traumatic synovitis					
MM-G	Normal	0	0	0	0
MM-G	RA (NV)	5120	10240	10240	10240
MM-G	RA (VB)	20480	20480	20480	20480
MM-G	RA (GS)	10240	10240	10240	10240
MM-G	RA (LB)	640	1280	320	640
MM-G	RA (AR)	1280	1280	1280	1280
Rheumatoid synovitis					
IP-H	Normal	0	0	0	0
IP-H	RA (AH)	5120	5120	40960	40960

single normal serum used as a medium component for a single cell strain at different transfer ages will sometimes yield individual HA synthesis rates deviating by 20 to 40 per cent from the mean performance value for that strain.

In the experiments recorded in Tables 3 and 4, it is important to note that sera to be compared were used simultaneously on cells from the same stock culture populations. Thus, the responding cells were of the same *in vitro age* and in the same biological condition at the onset of the test procedure.

Increased HA synthesis rates in the presence of rheumatoid serum usually were an arithmetic consequence of reduced cell division associated with unchanged or slightly augmented total HA formation in a culture. Is such a pattern specific for rheumatoid sera? Substances with general biologic toxicity might be expected to depress both cell division and specific macromolecular synthesis. Examination of Table 5 shows 3 examples of sera from patients with polycythemia rubra vera (sera 89, 104 and 127) where such a general suppression of cellular activity was observed. On the other hand, polycythemic sera 105 and 123 altered connective tissue cell function in much the same fashion as did rheumatoid sera.

Drugs taken by patients are not likely to be responsible for the common serum effects, especially since aspirin was the only drug common to all donors of rheumatoid serum. Addition of salicylic acid in amounts equivalent to blood levels of 10 to 20 mg. per cent failed to affect proliferative rate or HA synthesis in pilot experiments. Moreover, addition of hydroxychloroquin in medium concentrations of 1.0 to 3.0 μg./ml. did not alter fibroblast proliferation or HA formation.

Rheumatoid Factor Measurements

To examine rheumatoid factor in relation to the observed alterations in cellular function, the end point titer of rheumatoid factor was measured in unused medium and medium removed at intervals from growing cultures during a 7-day growth cycle. We interpreted the serologic data (Table 6) to show that no significant alteration in titer of rheumatoid factor occurred as a result of 48–72 hours incubation at 37 C with either "rheumatoid" or non-rheumatoid fibroblasts. In most cases the titers

Table 7.—*Residual Rheumatoid Factor in Fibroblasts Exposed to Rheumatoid Sera*

Cell strain MM (number of Cells × 10^6)	Donor of RA sera	R.F. titer: Whole sonicate	R.F. titer: Supernatant (post centrifugation)
14.0 × 10^6	Normal	1:2	0
10.0 × 10^6	N.V.	1:4	0
11.9 × 10^6	V.B.	1:4	0
11.3 × 10^6	G.S.	1:8	0
9.2 × 10^6	L.B.	1:2	0
10.0 × 10^6	A.R.	1:2	0

were identical following incubation with cells, with an occasional one-tube variation. The increase in titer measured when rheumatoid sera AH was placed on rheumatoid cells (I.P.) is of uncertain significance, although it may merely reflect an enhancing effect of mucopolysaccharide on rheumatoid factor measurement.

Attempts to detect R.F. bound to cultured connective tissue cells failed to yield significant titers. Neither whole cell sonicates nor the supernatant fluid following centrifugation exhibited activity detected by latex fixation (Table 7). Not only were we unable to detect disappearance of R.F. activity from culture media, or binding to cells, but inspection of Tables 3 and 6 suggests that the degree of "stimulation" of HA synthesis rate may have been *inversely* related to the titer of rheumatoid factor.

Intrinsic Viscosity Alterations in Hyaluronate

Hyaluronic acid synthesized by human articular cells in culture exhibits variable intrinsic viscosity (a parameter of molecular weight) which is related to the stage of the culture growth cycle.[7] Hyaluronate formed during the early (lag phase) portion of the growth cycle has the highest intrinsic viscosity, and this decreases markedly in medium changes corresponding to the growth and plateau portions of the cycle. As shown in Table 8, there was a tendency for cells supported by some rheumatoid sera to form HA of lower molecular weight when compared with sister cultures nourished by normal sera. Cells of normal origin (AM-II), traumatic origin (MM) and from rheumatoid sources (I.P. and Ma B) were similarly affected by sera from rheumatoid donors. In four instances the depression of molecular weight accompanied quantitative increases in total HA synthesis, but the experiment with strain MaB indicates that this was not a necessary relationship. Several rheumatoid sera had essentially no effect on the intrinsic viscosity of hyaluronate formed by cell strain MM.

Table 8.—*Effect of Rheumatoid Serum on Intrinsic Viscosity of Culture Produced H.A.*

Cell strain	Serum source	HA/flask (μg.)	$[\eta]$ at 1st medium change
AM II-G	Normal	64.0	43.7
AM II-G	RA-pool	101.0	26.7*
MM-E	Normal	62.0	45.2
MM-E	RA-(FD)	82.0	39.0
IP-G	Normal	58.0	43.1
IP-G	RA-(FD)	73.0	20.0*
MaB-F	Normal	80.4	28.0
MaB-F	RA-pool	71.0	19.8*
MM-G	Normal	116.0	33.6
MM-G	RA-(A.R.)	152.0	23.0*
MM-G	RA-(V.B.)	104.0	30.8
MM-G	RA-(L.B.)	106.0	31.1
MM-G	RA-(G.S.)	90.0	34.4
MM-G	RA-(N.V.)	80.0	38.8

*Indicates intrinsic viscosity measurements which were markedly lowered in the presence of rheumatoid sera when compared with the appropriate controls.

Discussion

The data suggest that rheumatoid sera tend to be relatively inhibitory to fibroblastic proliferation in comparison with normal sera prepared in the same fashion. It

is clear that there is not a commensurate depression of hyaluronic acid synthesis; in fact, some sera support an absolute increase in the total amount of hyaluronic acid formed by a culture, and in virtually all cases there is an increase in the amount formed per individual cellular unit. In 4 of 9, the RA sera appeared to be associated with formation of smaller HA polymers. This change in metabolic activity does not appear to involve rheumatoid factor, since we were unable to demonstrate loss of titer dependent upon incubation with the cells and equally unable to find agglutinating activity attached to the cellular fraction.

It would appear that there are humoral substances in the sera of patients with rheumatoid arthritis that are capable of modifying the proliferative and synthetic activities of human fibroblasts whether they be of normal or rheumatoid origin. Our data indicate that the factors in rheumatoid sera which alter connective tissue cell performance produce effects of very modest dimensions, and that these effects are not specific since they may be caused by polycythemic sera.

Summario In Interlingua

Sero rheumatoide deprime in vitro le proliferation de fibroblastos synovial e tende a intensificar le formation de acido hyaluronic in le cellulas individual. In terminos quantitative, le effectos observate non esseva grande. Apparentemente illos non es specific pro un morbo particular e non monstra un correlation con le titro de factor rheumatoide mesurate per fixation a latex. Es describite un meliorate methodo pro mesurar mucopolysaccharida acide in medios histocultural.

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