PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 594–598 (1975)

Rubella and Rheumatoid Arthritis: Hyaluronic Acid and Susceptibility of Cultured Rheumatoid Synovial Cells to Viruses[1] (38859)

ROBERT L. PATTERSON, DAVID A. PETERSON, FRIEDRICH DEINHARDT, AND FRANCIS HOWARD

Departments of Microbiology and Orthopedic Surgery, Rush-Presbyterian-St. Luke's Medical Center and Department of Microbiology, University of Illinois Medical Center, Chicago, Illinois 60612

The frequent association of arthritis either with natural rubella virus (RV) infections or with vaccination by attenuated, live RV and seroepidemiological studies indicate that some causal relationship may exist between RV and rheumatoid arthritis (RA). Several studies designed to probe this relationship examined the susceptibility *in vitro* of RA synovial cells to various viruses. Person *et al.* (1) and Parker *et al.* (2) found that RA synovial tissue cultures were susceptible to RV, Newcastle disease virus (NDV), and vesicular stomatitis virus (VSV), whereas Ford (3) reported that high-passage RV vaccine (HPV-77) produced no cytopathic effect (CPE) in cultured RA and non-RA synovial cells and Grayzel and Beck (4, 10), Smith (5), Smith *et al.* (6, 7), and Patterson *et al.* (8) found that RA synovial cells were resistant to RV and NDV. Although viral interference through a preexisting chronic or latent infection of the synovial cells was an attractive explanation for the resistance to infection with RV and NDV, it did not explain the discrepancy in results from different laboratories. It seemed possible that hyaluronic acid (HA) produced by the RA synovial cells might itself have changed the susceptibility of RA cells to various viruses and this study examined this possibility.

Materials and Methods. Tissue cultures. Rheumatoid synovial specimens were obtained from patients undergoing surgical synovectomy; nonrheumatoid synovial specimens were obtained from two stillborn fetuses. Tissues was trypsinized and grown in RPMI 1640 medium with 10% inactivated fetal calf serum, 100 units/ml penicillin, and 50 μg/ml streptomycin. After monolayers were established medium was changed to basal medium of Eagle's (BME) in Earle's balanced salt solution (E) supplemented with 2% fetal calf serum and antibiotics. The primary cell cultures contained fibroblasts, macrophages, and some undifferentiated mesenchymal cells; upon subculturing the cell population became fibroblastic.

Hyaluronic acid. Supernatants from RA and normal synovial cell cultures were examined for HA by Cohen's qualitative method (11). Cultures were rinsed three to five times with Hanks' balanced salt solution (HBSS) to remove serum, refed with a serum-free medium composed of equal amounts of BME-E and medium 199 plus antibiotics, and incubated for 2–3 days. Thereafter 2.0 ml of tissue culture fluid was mixed thoroughly with 0.15 ml of 7 *N* acetic acid at 22–24° and observed for mucoid precipitates. Precipitates were scored according to Cohen's original description from the condition of the mass formed after the addition of acetic acid: 3+ "tight ropy mass," 2+ "softer mass with some shreds in solution," 1+ "clumped flecks of mucin suspended in a cloudy solution," and 0 "no detectable precipitate." To isolate hyaluronic acid, 200–250 ml of tissue culture fluid collected from cultures were weighed and then chilled at 5° for 15–20 min. Glacial acetic acid was added to a final volume of 1%, the mixture was stirred, and allowed to stand for 20 min. The precipitates were centrifuged at 200*g* for 15 min at room temperature, the supernatant was decanted, and the HA precipitate was washed three times with cold distilled water and dissolved in 2.0 ml, 0.1 *M* NaCl, 0.1 *M* $NaHCO_3$ buffer, pH 8.1. The viscosity of the HA solution was measured with a Zeitfuchs cross-arm viscometer (12).

[1] This work was supported in part by the Arthritis Foundation, Illinois Chapter, and the Jules J. Reingold Trust Fund.

Virus strains. Clinical isolates of rubella virus (SV395) and Coxsackie A9 virus (SS-1972), Indiana strain of VSV, and Kan-Manhattan strain of NDV were used. Stock viruses were grown in normal synovial cells and stored at −65°.

Culture susceptibility. The susceptibility of synovial cells was determined by the inoculation of 500–5,000 $TCID_{50}$/ml each of RV, VSV, and NDV into four replicate cultures of RA and normal synovial cultures. After absorption for 2 hr at 37°, the inoculum was removed, the cultures were rinsed once with HBSS, and refed with maintenance medium. All cultures were examined for CPE at 2-day intervals for 14 days and scored as + (distinct CPE leading to destruction of all cells), ± (distinct CPE but not destruction of all cells), and − (no CPE).

The effect of HA on the susceptibility of RNA synovial cells was determined by two approaches; (1) the effect of hyaluronidase on susceptibility and (2) the change in susceptibility after exposure to HA. In the first approach, 300 U of hyaluronidase were incubated with RA and normal synovial cultures for 2 hr at 37°, the enzyme was removed, cultures were rinsed seven times with HBSS, and inoculated with 5000 $TCID_{50}$ VSV or 100 $TCID_{50}$ RV in HBSS. After 2-hr absorption the inoculum was removed, cultures were rinsed once with HBSS, and refed with maintenance medium. Untreated cultures served as controls. At various times after infection, supernatant was removed, additional maintenance medium was added, the cells were frozen and thawed three times, the supernatants and freeze-thawed cells were titrated for VSV and RV in primary rhesus monkey kidney cultures (RhMK) and primary African green monkey kidney cultures (GMK), respectively. In the second approach a normal synovium cell culture (S27) was exposed for 2 hr to HA isolated from the RA-S2 cell line. Cells were rinsed seven times with HBSS and inoculated with 5000 $TCID_{50}$ of VSV and processed as above. Normal, untreated S27 synovial cells served as controls.

Results. In preliminary experiments the culture fluids of 28 RA, one osteoarthritis, and three normal synovial cell cultures were tested for HA (Table I) and the viscosity of the supernatants of three synovial cells lines (RA-SO2 TC 12; RA-S2 TC 17; Nor-S27 TC 17), of maintenance medium, and of triple-distilled water was determined (Table II). HA from the two RA cell lines was more viscous than the supernatant from the normal synovial cell line, maintenance medium, or triple-distilled water. After treatment of

TABLE I. DETECTION OF HYALURONIC ACID IN TISSUE CULTURE FLUIDS OF SYNOVIAL CELL CULTURES.

Designation[a]	Passage[b]	Hyaluronic acid[c]
RA-S01	8	0
RA-S02	8	1+
RA-S03	6	2+
RA-S03	56	0
RA-S04	12	2+
RA-S05	1 and 6	0
RA-S2	13	2+
RA-S07	9 and 12	1+
RA-S11	9	2+
RA-S13	8	1+
RA-S15	7	1+
RA-S17	6	1+
RA-S20	5 and 9	1+
RA-S21	9	1+
RA-S22	3	1+
RA-S23	9	1+
RA-S24	9	1+
RA-S25	18	2+
RA-S26	2	2+
RA-S28	1	1+
RA-S29	1	1+
RA-S30	1	2+
RA-S32	1	1+
RA-S37	1	1+
RA-S42	1	1+
RA-S44	1	1+
RA-S48	1	2+
RA-S51	2	2+
Ost-S14	10	2+
Nor-S27	13	0
Nor-S31	1	0
Nor-S36	1	0

[a] RA = rheumatoid arthritis, Ost = osteoarthritis, Nor = nonrheumatoid arthritis.

[b] Number of culture passage examined for HA.

[c] Precipitin results recorded on a 0 to 3+ scale; 0 = no visible reaction, 1+ = soft shredded mass in a turbid solution, 2+ = firm mass with some shredding, 3+ = tight ropy mass.

TABLE II. VISCOSITY OF SYNOVIAL TISSUE CULTURE FLUID.

	Untreated		Treated[a]	
	Retention[b] time (sec)	Viscosity[c]	Retention time (sec)	Viscosity
RA-S02 TC 12	143	1.71	73	0.88
RA-S2 TC 17	158	1.89	75	0.91
Nor-S27 TC 17	80	0.96	75	0.90
RPMI 1640- 2% fetal calf serum	72	0.86	72	0.86
Distilled water	69	0.83	71	0.85

[a] Treated with 250 units of hyaluronidase.

[b] Each value is an average of three successive measurements of the sample by a Zeitfuch's cross-arm viscometer.

[c] Viscosity is calculated by multiplying the efflux time by the viscometer standard (corrected for distilled Chicago water).

the RA cell culture supernatants with 250 U of hyaluronidase, the viscosity of the fluids was comparable to that of distilled water and maintenance medium, neither of which exhibited any change in viscosity after treatment with hyaluronidase.

The effect of HA on the susceptibility of RA and normal synovial cell cultures to virus infection was examined in the following experiments. First, the viral susceptibility of 15 synovial cultures to RV, VSV, and NDV was determined (Table III). In many cultures a distinct CPE was seen which did not progress to destruction of all cells in the culture; in some instances cultures which showed an initial, partial CPE recovered and appeared completely normal at the end of the 14-day observation period. The role played by HA in the susceptibility of synovial cells to RV was examined by pretreatment of four RA synovial cell cultures, one culture of carpal tunnel syndrome (CT), and two normal synovial cell cultures with hyaluronidase (Table IV). Comparison of RV titers in RA and CT synovial cells untreated or treated with hyaluronidase shows that viral replication occurred after enzyme pretreatment of the cells.

A more detailed growth curve of viral replication in treated and untreated RA cells is given in Fig. 1. The cell line RA-S2, a high producer of HA, was unaffected by VSV when untreated, but after cells had been exposed to the depolymerizing effects of hyaluronidase, VSV replicated in them as efficiently as in normal synovial cells.

TABLE III. SUSCEPTIBILITY OF SYNOVIAL CELLS TO RV, VSV AND NDV.[a]

Synovial cell designation[b]	Hyaluronic acid	Virus inocula: RV 500 $TCID_{50}$	VSV 5000 $TCID_{50}$	NDV 500 $TCID_{50}$
RA-S01 TC 7	0	±	+	+
RA-S02 TC 7	1+	±	−	±
RA-S03 TC 9	0	±	+	+
RA-S2 TC 4	2+	−	−	±
RA-S07 TC 4	1+	−	±	−
RA-S13 TC 2	1+	−	±	−
RA-S20 TC 6	1+	−	±	−
RA-S21 TC 4	1+	−	+	NT
RA-S22 TC 5	1+	−	−	−
RA-S23 TC 6	1+	+	+	NT
RA-S25 TC 12	2+	−	+	−
RA-S26 TC 6	2+	−	±	−
RA-S28 TC 9	1+	NT	±	−
RA-S29 TC 6	1+	NT	−	±
RA-S30 TC 8	2+	NT	+	−
Nor-S27 TC 18	0	+	+	+
Nor-S31 TC 21	0	+	+	+

[a] Determined by viral cytopathogenic effect (CPE): + = CPE, resulting in destruction of all cells, ± = CPE with only partial destruction of culture with many cells surviving, − = No CPE, NT = not tested.

[b] RA = rheumatoid arthritis, TC = tissue culture passage used for test, Nor = nonrheumatoid arthritis.

Confirmation of the effect of HA on viral multiplication is shown in Fig. 2. VSV replicated efficiently in normal synovial cells but when HA was added to resting cultures for a

TABLE IV. EFFECT OF HYALURONIDASE TREATMENT ON RV TITERS IN RA SYNOVIAL CELLS.

Cell line	Treatment: Hyaluronic acid	Hyaluronidase[a]	None
RA-S2 TC 8	2+	5×10^{4}[b]	$<1 \times 10^{1}$
RA-S20 TC 10	1+	5×10^{4}	5×10^{4}
RA-S25 TC 8	2+	5×10^{4}	$<1 \times 10^{1}$
RA-S26 TC 9	2+	5×10^{4}	$<1 \times 10^{1}$
CT-S59 TC 10[c]	1+	5×10^{3}	$<1 \times 10^{1}$
Nor-S77 TC 4	0	5×10^{4}	5×10^{4}
Nor-S27 TC 6	0	5×10^{4}	5×10^{4}

[a] Cells treated with 300 units of hyaluronidase before inoculation of RV.

[b] RV titer/ml determined by interference of 1000 $TCID_{50}$ Coxsackie A9 virus.

[c] CT = Carpal tunnel syndrome.

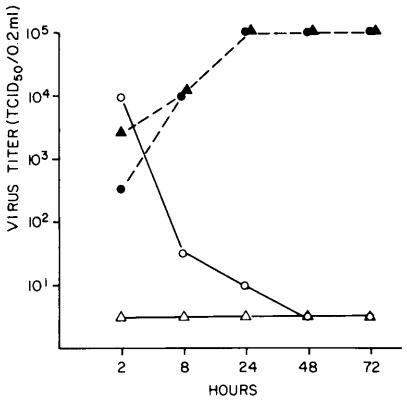

FIG. 1. Multiplication curves of VSV in untreated RA synovial cells (RA-S2) producing inhibitory amounts of hyaluronic acid and in RA-S2 cells treated with 300 U hyaluronidase before inoculation with 1000 $TCID_{50}$ VSV. VSV was titrated in RhMK. ○——○ Supernatant, untreated RA S2; △——△ cells, untreated RA S2; ●——● Supernatant, hyaluronidase-treated RA S2; ▲——▲ cells, hyaluronidase-treated RA S2.

minimum of 2 hr, VSV was detected in the medium only until 8 hr after infection, demonstrating that virus did not multiply in the cells because HA blocked adsorption and/or penetration.

Discussion. The inability of certain viruses to infect RA synovial cells led to the belief that these cells were chronically or latently infected by a virus (4–6). In contrast, others

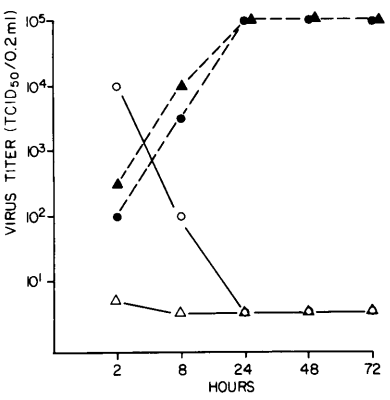

FIG. 2. The effect of partially purified hyaluronic acid on the multiplication curve of VSV in normal synovial cells (S27), negative for hyaluronic acid production. Both sets of cultures were treated with 300 U of hyaluronidase followed by the addition of media to the untreated set and media plus 100X hyaluronic acid to the other and both were infected with 1000 $TCID_{50}$ VSV. VSV production was titrated in RhMK. ○——○ VSV titer of supernatant after cells were pretreated with hyaluronic acid; △——△ VSV titer after cellular disruption by freeze-thawing; cells were pretreated with hyaluronic acid before infection; ●——● VSV titer of supernatant of untreated cells; ▲——▲ VSV titer after cellular disruption by freeze-thawing of untreated cells.

(1, 2) found that certain viruses replicated freely in RA synovial cells without any indication of inapparent infection. In a series of experiments, some of which were presented previously in preliminary form (8), we demonstrated that certain viruses were unable to infect RA synovial cell cultures because the cells were covered with appreciable amounts of HA; the resistance of the rheumatoid synovial cell cultures was due not to viral interference but to excessive production of HA by the cultured cells. The specificity of the inhibitory effects of HA was demonstrated both by adding HA to normal synovial cells, preventing these cells from absorbing VSV, and conversely by depolymerizing HA by the addition of hyaluronidase to RA cells rich in HA thereby allowing the absorption and penetration of VSV and RV. Similar results of increased susceptibility of RA and non-RA synovial cells after treatment with hyaluronidase were reported recently (9).

Studies to determine whether RV or any other viruses play a role in the etiology of some forms of rheumatoid arthritis are continuing.

Summary. Synovial cell lines were established from patients with rheumatoid arthritis (RA) and from normal human embryos. High levels of hyaluronic acid (HA) were produced by some RA cell lines, some of which were partially or completely resistant to infection with Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), and rubella virus (RV). Normal fetal synovial cells lines were susceptible to NDV, VSV, and RV. Infection with virus became possible after treatment of RA cells with hyaluronidase to depolymerize HA, and HA prevented infection of normal synovial cells with VSV. These results provide evidence that HA and not chronic or latent viral infection is responsible for the lack of susceptibility of RA synovial cells to certain viruses.

1. Person, D. A., Rawls, W. E., and Sharp, J. T., Proc. Soc. Exp. Biol. Med. **138,** 748 (1971).
2. Parker, M. D., McCollum, D. E., and Kerby, G. P., Arthritis Rheum. **15,** 275 (1972).
3. Ford, D. K., Arthritis Rheum. **15,** 313 (1973).
4. Grayzel, A. I., and Beck, C. J., Exp. Med. **131,** 367 (1970).
5. Smith, C. A., J. Exp. Med. **134,** 306 (1971).
6. Smith, C. A., Janis, R., Habermann, E., and Hamerman, D., Arthritis Rheum. **13,** 349 (1970).
7. Smith, C. A., Hamerman, D., Janis, R., and Habermann, E., Ann. Rheum. Dis. **33,** 173 (1974).
8. Patterson, R., Howard, F., and Deinhardt, F., Clin. Res. **21,** 878, (1973).
9. Clarris, B. J., Fraser, J. R. E., and Rodda, S. J., Ann. Rheum. Dis. **33,** 240 (1974).
10. Grayzel, A. I., and Beck, C., Proc. Soc. Exp. Biol. Med. **135,** 496 (1971).
11. Cohen, A. S., *in* "Laboratory Diagnostic Procedures in Rheumatic Diseases" (A. S. Cohen, ed.), p. 2. Little, Brown, Boston (1967).
12. Cannon Instrument Company, State College, PA.

Received Feb. 10, 1975. P.S.E.B.M., 1975, Vol. 149.