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Characteristics of an Antigen Reactivity in Synovial Fluids and Its Association with Rheumatoid Arthritis (39798)

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Introduction. The synovial lining cells of joint tissue undergo hyperplasia in rheumatoid arthritis (RA) and several other inflammatory joint conditions. Synovial fibroblasts derived from RA individuals and grown in tissue culture differ from non-RA synovial fibroblasts in a number of growth and biochemical criteria (1, 2). The RA synovial fibroblasts have shorter life spans *in vitro*, a longer generation time, and a decreased density of cell numbers at confluence; they also differ from non-RA synovial fibroblasts in their rate of hyaluronic acid formation, their response to hydrocortisone, and their content and metabolic responses to a connective tissue activating peptide. These characteristics, which may be common to RA synovial fibroblast cell lines and distinct from non-RA cell lines, constitute substantial evidence of a difference in genetic or acquired expression between the two types of cell strains. In addition, RA synovial fibroblasts differ from non-RA fibroblasts in their potential infectivity by rubella virus because such fibroblasts, when fused with non-RA cell lines, can confer resistance to lytic infection with this virus (3, 4). These results, however, have not been confirmed in other laboratories (5, 6), and it has been suggested that the observed resistance may have been due to an increased synthesis of hyaluronic acid (7).

It is possible that either a qualitative or quantitative difference in structural, soluble, or secreted components will also differentiate the cells of normal and pathologic origin. Antisera were prepared in this laboratory to RA synovial fibroblasts grown *in vitro* to test the possibility that a structure unique to the RA synovial fibroblast might be described by its antigenic reactivity and delineated by appropriate absorption methods. The antisera react with a number of antigen sources: live RA and non-RA synovial and dermal fibroblasts, the supernatants from fibroblast cell extracts, human serum and plasma, and synovial fluids. In immunodiffusion analyses the human sera, plasma, and synovial fluids gave single precipitin lines against the anti-RA synovial fibroblast sera, which fused in all cases with lines of identity. However, when the antigen reactivities of synovial fluids were examined by the immunoelectrophoresis (IEP) technique, a wing or long (extended) precipitin arc pattern was consistently obtained which was associated by statistical analyses of the results with the fluids from patients with RA.

Materials and Methods. Synovial fibroblasts were grown in tissue culture by explanting synovial tissue obtained from RA patients at the time of synovectomy. The two tissues used for explants were knee synovia of female patients, both patients having had a history of RA extending over several years. The cells were maintained in CMRL 1066 media (Grand Island Biological Co.; GIBCO) with 20% fetal calf serum (FCS; GIBCO) in a humidified atmosphere of 5% CO_2–95% air at 37° and were subcultured using 0.25% viokase (GIBCO). The cells used for immunizations were scrape-harvested from flasks or roller bottles, washed with phosphate-buffered saline (PBS; pH

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7.4), suspended in 1 ml of PBS, and mixed with an equal volume of adjuvant. The first immunization was done with Freund's complete adjuvant and subsequent immunizations with incomplete adjuvant. Approximately 2 to 3 $\times$ 10^7 cells were used to immunize New Zealand white rabbits by multiple subcutaneous and intraperitoneal injections. Postimmunization sera were collected, heat inactivated at 56° for 30 min, titered against live tissue cultured cells by indirect immunofluorescence assays, and used in the tests described below. After the third and fourth immunizations of the same animal, antisera of sufficient antibody titer were obtained.

IEP analyses were performed using 0.8% agarose plates with 0.0385 *M* sodium barbital buffer (pH 8.2) plus 0.01% sodium azide. Electrophoreses of antigen preparations were carried out for 1 hr at 8 mA/plate, and 4 V/cm. After electrophoresis, the antiserum was applied to the troughs and precipitin band formation was noted for up to 48 hr. The plates were then washed extensively with 0.03 *M* NaCl, stained for protein with triple stain (9), and photographed. The periodic acid–Schiff (10) and fat red 7B reagents (11) were also used to stain some of the IEP plates.

The synovial fluids had been collected for diagnostic purposes at the Arthritis Clinics of Northwestern University and The University of Michigan Medical Schools and associated hospitals. All RA patients fulfilled established diagnostic criteria for definite or classical rheumatoid arthritis (12).

Concentration of synovial fluids or antisera was done in B15 Minicon chambers (Amicon Corp.). The IgG fraction of rabbit antiserum was isolated by the method of Reif (8). The enzyme hydrolases used were obtained from Sigma Chemical Co. or Worthington Biochemicals and the antisera to human serum components were purchased from Cappel Laboratories and Miles Laboratories. An IgM rheumatoid factor preparation which had activity to human and rabbit IgG was obtained from Dr. M. Floyd. Carcinoembryonic antigen was a gift from Dr. J. Tomita and glycophorin from Dr. J. Garvin. Bovine proteoglycan preparations were provided by Drs. Breen, Weinstein, and Eisenstein.

Results. As shown in Fig. 1, IEP analysis of some of the RA fluids resulted in the formation of a wing precipitin pattern. One arc of the wing remained over the antigen well (in the same position as immunoglobulin M under these IEP conditions) and the second arc moved anodally to the β–α_2 region. Because the precipitin lines do not cross at the juncture of the two arcs, the two molecular species that are revealed must be identical or cross-reactive in their antigenic determinants. Of the 49 RA synovial fluids tested, 25 gave this type of pattern. There were minor variations in the intensities of the two bands, indicating that the concentrations of antigen varied in the samples. Nineteen other RA synovial fluids gave a long precipitin arc extending over the antigen well to the β–α_2 region. The intensity of the long precipitin arc varied, giving an indication that the two antigen species, as seen in the wing precipitin pattern, are present but not as clearly resolved under these IEP conditions. Of the five remaining RA synovial fluids not giving either type of pattern, three gave a single precipitin arc located slightly toward the anode. The other two gave no detectable precipitin arc. Five of thirteen (38%) non-RA (two traumatic arthritis, two osteoarthritis, one chondrocalcinosis) synovial fluids gave the wing precipitin arcs; seven (one traumatic arthritis, one ankylosing spondylitis, one gout, four osteoarthritis) gave a single precipitin band, which remained over the antigen well, and one (psoriatic arthritis) gave no detectable precipitin arc. Statistical analysis of the presence of the wing and long precipitin patterns in the RA and non-RA populations of synovial fluids by the χ^2 test is consistent with the wing and long IEP patterns association with RA ($P < 0.005$).

Efforts were made to determine whether a more anodal antigenic species may have been present in non-RA fluids which gave a single precipitin arc but may not have been detected by the antisera. Fivefold concentration of the synovial fluids which gave either a single or wing precipitin pattern did not reveal an additional antigen. Also, the antisera could be diluted up to 1:10 and the wing precipitin pattern was still clearly dis-

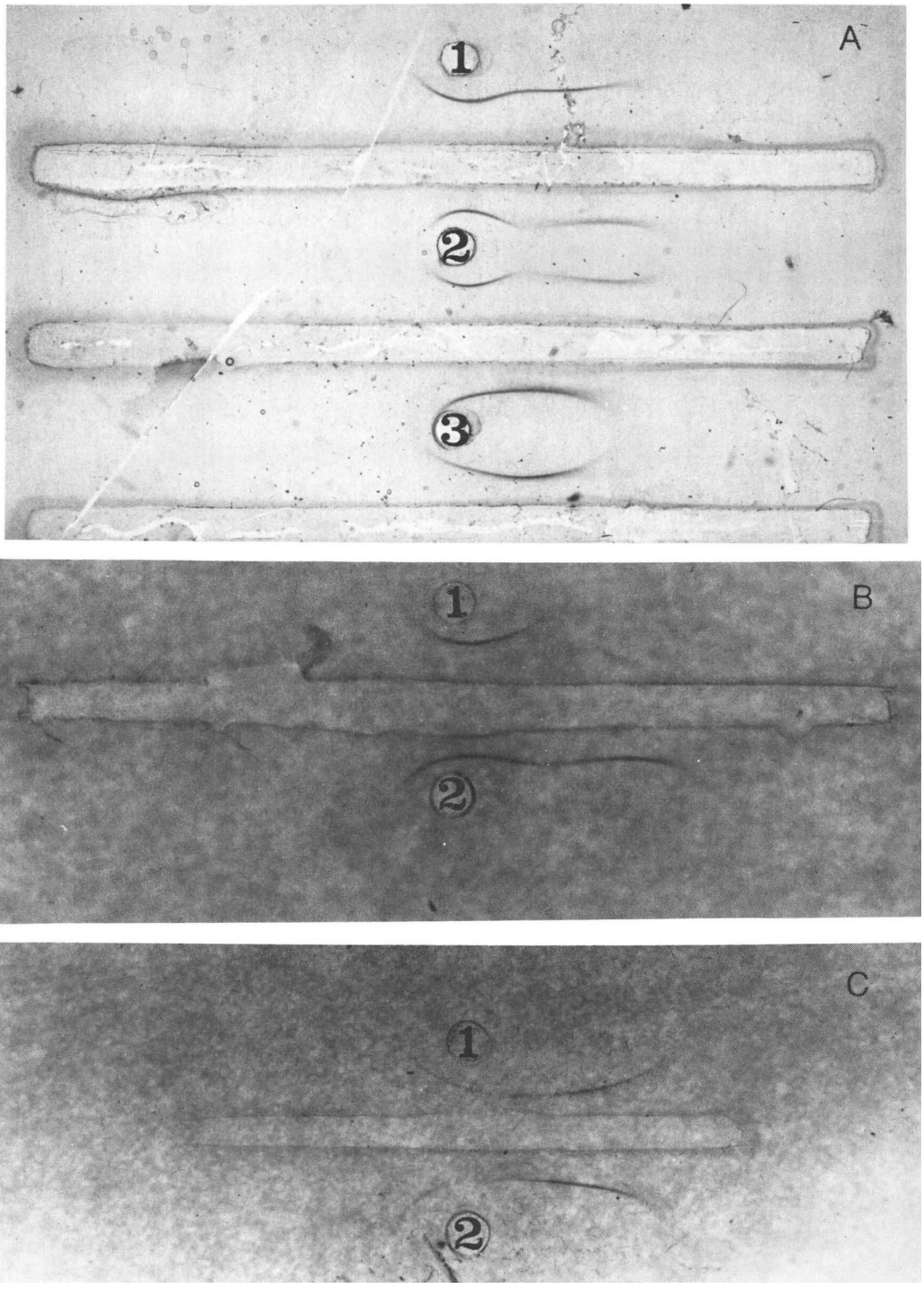

FIG. 1. Immunoelectrophoretic analyses of synovial fluids were done in agarose gels as described in the text. The anode is to the right; wells contained the particular synovial fluid and troughs contained anti-RA synovial fibroblast serum. (A) Wells 1 and 2 contained RA synovial fluids giving the wing precipitin pattern; differences in the intensities of the two components of the wing are clearly seen in sample 2. Well 3 contained synovial fluid from a patient with osteoarthritis and shows a single precipitin arc with a slight anodal mobility. (B) Antigen wells 1 and 2 contained synovial fluids from patients with osteoarthritis and RA, respectively, the former sample giving a single precipitin arc remaining over the antigen well. (C) Wells 1 and 2 both contained RA synovial fluids which gave long (extended) precipitin arcs. The intensities of the precipitin arcs are stronger in their more anodal portions.

cernible. In addition, fourfold concentration of antiserum resulted in no additional precipitin arc formation. Using the same concentrated antiserum against a fluid which had given a long precipitin arc resulted in a more distinct precipitin pattern. Thus, the long precipitin arcs may indeed be a variation of the wing pattern rather than representative of any different antigen specificity.

Characteristics of the antisera and reactive antigens were further investigated. Antisera from rabbits immunized with RA synovial fibroblasts from two different individuals gave identical precipitin patterns in the IEP analyses; preimmune rabbit sera did not react with synovial fluids. Furthermore, the antisera from the one animal used most extensively gave identical precipitin patterns regardless of the week postimmunization when a particular antiserum was obtained. An immunoglobulin G fraction of an antiserum gave the same precipitin patterns as the whole antiserum. The antisera reacted with a component of FCS in which the RA synovial fibroblasts were grown, however, absorption of antisera with FCS did not diminish the reactivity to synovial fluids. The synovial fluids tested varied in pH from 6.8 to 8.8 when studied, but the pH differences did not affect the electrophoretic mobility for the fluids giving a single, long or wing precipitin arc. The synovial fluid reactive antigen(s) contains carbohydrate since the precipitin band was stained with the periodic acid–Schiff reagent. No lipid components were detected with the fat red 7B reagent. The antigen's mobility in electrophoresis was affected by a number of proteases and the antigenic reactivity was completely destroyed by treatment of synovial fluids with pepsin and alkaline protease. Enzymatic hydrolysis of synovial fluids with testicular hyaluronidase, collagenase, RNase, and DNase had no effect on the antigenic reactivity.

Using purified preparations of human serum or plasma, connective tissue antigens, or specific antisera to these antigens, the reactive antigen(s) in synovial fluids was shown not to have any identity either by immunodiffusion or IEP analyses with any of the following: immunoglobulins G, M, or A, rheumatoid factor, haptoglobin, fibrinogen, transferrin, ceruloplasmin, α_2-macroglobulin, α_1-antitrypsin, C-reactive protein, α_1- or β-lipoproteins, C3 and C1q components of complement, α_1-acid glycoprotein, collagen, plasminogen, α_2-AP-glycoprotein, carcinoembryonic antigen, or glycophorin (human erythrocyte glycoprotein).

The antigenic reactivity described here is different from the antisera reactivities to cartilage protein–polysaccharides described by Sandson *et al.* (13) and to the species-specific component of cartilage fractions (14). The latter reactivity was found in non-RA arthritic fluids and a diminished reactivity was noted in RA synovial fluids. In contrast, the anti-RA synovial fibroblast sera reacted in immunodiffusion against all RA and most non-RA synovial fluids with approximately equal intensities of precipitin formation. Also, the anti-RA synovial fibroblast sera reactivities to synovial fluids were not diminished following absorptions with bovine proteoglycan subunit and aggregate fractions from skin, nasal septa, or intervertebral discs, or with human umbilical cord hyaluronic acid.

The anti-RA synovial fibroblast sera reactivities are also different from those described for antisera reactivities to hyaluronate–protein (15) and the inter-α trypsin inhibitor found in synovial fluids (16). The latter reactivities cannot be detected by immunodiffusion unless the synovial fluids are first digested with hyaluronidase, whereas the anti-RA synovial fibroblast sera react with synovial fluids without prior hydrolase treatments. The electrophoretic mobilities of these reactivities are also different. In addition, hyaluronidase treatment of synovial fluids did not affect this unusual synovial fluid antigen, and mixing anti-RA synovial fibroblast sera with hyaluronate did not diminish its reactivity with synovial fluids.

Normal synovial fluid consists of hyaluronate–protein and the lower-molecular-weight components of plasma (e.g., albumin, transferrin, immunoglobulin G). The protein content is less than 2.5% (17). In inflamed joints (as seen in RA, acute rheumatic fever, systemic lupus erythematosus, gout, and other forms of synovitis), the synovial fluid contains greater amounts of the

lower-molecular-weight components and additional proteins of larger molecular weight including β_2-macroglobulin, β-lipoprotein, and α_2-macroglobulin (18). Also, the concentration of hyaluronate is reduced to one-third to one-half the concentration in normal synovial fluids. The possibility that the extra precipitin arc associated with RA synovial fluids was derived from the influx of a serum component during inflammation was examined by testing the synovial fluid samples for the presence of known serum components of large molecular weight, β-lipoprotein and α_2-macroglobulin. These two serum components were found in all but two of the samples used in this study (i.e., both RA and non-RA synovial fluid samples). Therefore, their presence did not correlate with the described RA-associated antigenic reactivity.

Discussion. The synovial fluid antigen described in these studies is a protein, as shown by the effects on electrophoretic mobility by a number of proteases, and is possibly a glycoprotein, as indicated by its reaction with periodic acid and Schiff's base formation. The antigen does not have any characteristics of previously described components of synovial fluids. Considering that the antisera used to detect the antigen were produced in response to synovial fibroblasts, the derivation of the antigen may be from synovial lining cells, either as fibroblast-secreted products or as a consequence of fibroblast cellular components spilled into the synovial fluid at cell death. The observation that the reactive antigens in human serum, plasma, and synovial fluids of RA and non-RA origin all formed precipitin bands without spurring in double immunodiffusion indicates that the antigens from the different sources are not substantially different qualitatively. Also, the antiserum could be absorbed with human serum or plasma and the reactivity to synovial fluids was substantially reduced. For this reason the synovial fluid antigen may also be derived in part from plasma. If the latter pertains, greater quantities of the antigen may enter the synovial fluid with increasing duration or intensity of inflammation.

The distinguishing feature of the IEP precipitin patterns was the anodal portion of the wing and extended precipitins; the portion of the precipitin arcs common to all the reactive synovial fluids remained over the antigen well. The anodal portion of the precipitin arcs could result from a single antigen species which had sustained partial enzymatic hydrolysis. Treatment of synovial fluids which gave a single precipitin arc with a number of proteases resulted in an altered electrophoretic mobility but did not lead to a wing precipitin arc pattern. Also, examination of a number of other synovial fluid components using antisera specific to human serum or plasma constituents did not reveal altered precipitin arc patterns as compared to those obtained with human serum. Therefore, any hydrolase giving the wing precipitin arc must have a different specificity from those studied. In addition, incubation of a fresh RA synovial fluid with a non-RA synovial fluid which gave a wing and a single precipitin arc, respectively, did not result in an increase in the intensity of the more anodal portion of the precipitin arc as compared to the nonincubated mixture of synovial fluids. However, hydrolysis of the synovial fluid antigen, which could be either tissue or plasma derived, could have occurred by enzymatic action in synovial tissue. Another possibility is that the two forms of the antigen may be due to two distinct molecules which are cross-reactive or share identical antigenic determinants. Also, the antigen reactivities may represent different polymeric forms of the antigen which differ in effective ionic charge and, therefore, in electrophoretic mobility.

The wing or extended precipitin arc patterns were found with RA synovial fluids with a 2.5 greater frequency than with non-RA fluids. The χ^2 test is an appropriate analysis for these types of data and establishes a certain probability that the precipitin pattern association with RA is not due to chance. There were insufficient numbers of other diagnostic criteria to attempt correlations with the IEP patterns at this time.

At present there is no particular feature, antibody reactivity, or antigen which can be considered as unique to any one of the connective tissue disorders. In these studies as well, although 90% of RA fluids are "positive" by IEP analyses, 38% of non-RA sam-

ples were also positive. As discussed by Bell *et al.* (19), such results may be expected because the connective tissue diseases are characterized by a spectrum of common features, some of which predominate for a particular disease.

Further studies are in progress to ascertain whether antisera to normal synovial fibroblasts will react with synovial fluids in a similar or different manner from the anti-RA synovial fibroblast sera. Also, attempts are being made to absorb antisera to a specificity for the more anodal portion of the precipitin arcs such that quantitations of the reactive antigens in synovial fluids can be done.

Summary. When antisera to RA synovial fibroblasts were reacted with synovial fluids in immunoelectrophoretic analyses, 90% of RA fluids gave a wing or extended precipitin arc pattern. In contrast, 38% of non-RA fluids gave the same types of patterns. The characteristics of the antigen are different from other previously reported synovial fluid components or antigens, and the antigen appears to be a glycoprotein.

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