RHEUMATOID SYNOVIOCYTES SECRETE A CHEMOATTRACTANT FOR MONOCYTES

by

Richard H. Edelson

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ABSTRACT:

Monocyte/macrophages in the synovium of patients with rheumatoid arthritis (RA) actively contribute to joint disease, but the mechanisms for their recruitment into joint tissue are unknown. We have established synoviocyte cultures from 5 patients with RA, 4 with degenerative joint disease (DJD) and 4 without significant joint disease (NL).

In a modified Boyden chamber assay, a 1% concentration of serum-free conditioned medium from RA synoviocytes induced significantly more migration than that induced by conditioned medium from NL synoviocytes. Checkerboard assays on supernatants from 4 of 5 RA cultures indicated chemotactic factor(s) were present while chemotactic factors were absent in all supernatants from cultures of NL synoviocytes. Conditioned medium from DJD synoviocytes also induced significantly more migration. However, conditioned medium from DJD synoviocytes generally induced chemokinesis (maximum migration independent of a stimulus gradient), not chemotaxis. Chemotactic activity from 1 RA culture was stable at 100 degrees C for 10 minutes or at pH 3.0 for 30 minutes, but was diminished after incubation with trypsin (suggesting that the chemotactic factor is a protein). Migratory

activity from RA conditioned medium but not DJD conditioned medium was recovered in several fractions from HPLC anion exchange chromatography and in 2 fractions from gel filtration chromatography corresponding to a molecular weight between 158,000-670,000 dalton.

The secretion of chemotactic activity by synoviocytes could contribute significantly to the pathogenesis of rheumatoid arthritis.

INTRODUCTION:

A leukocytic infiltrate is a hallmark of inflammation. Mobile phagocytes, which include polymorphonuclear leukocytes (PMN's) and monocytes, actively contribute to the process of inflammation by several methods. These include a) secretion of enzymes such as collagenase, elastase, acid hydrolases, and lysozyme (1); b) synthesizing lipid mediators of inflammation such as prostaglandins and leukotrienes (2); and c) releasing toxic oxygen radicals (1,16).

Monocytes and PMN leukocytes are capable of recognizing discrete chemical stimuli and migrating towards these stimuli. This cellular migration directed towards a chemoattractant along a concentration gradient is called chemotaxis (3). Chemokinesis, in contrast to chemotaxis, is maximum random nondirectional migration independent of a stimulus gradient. Substances which are known to be chemotactic for leukocytes include: complement degradation products (most notably C5a), leukotriene B4, platelet derived growth factor, fibronectin, interleukin 1, metenkephalin, beta-endorphin, substance P, platelet activating factor, elastin-derived peptides, collagen and collagen fragments, crystal induced chemotactic factor, bacterial products such as N-formylated methionyl peptides, and a

fibroblast produced factor (2,3,4,5,6,7,8,9,10,11). The relative contribution of these mediators to inflammatory processes in vivo is largely unknown.

The molecular and cellular responses of leukocytes to chemotactic factors has been, and is currently being closely studied. Chemotactic gradients are initially recognized by the binding of chemoattractants to specific cell surface receptors (2,12). Following binding to receptors, the initial morphological response of PMN's and mononuclear leukocytes to chemoattractants is a change in shape from round to an elongated, polarized configuration. This response involves the cytoskeletal rearrangement of actin microfilaments and is apparently mediated by calcium and cyclic nucleotides (2,3,13,14). The actin microfilaments are responsible for locomotion of the cell, whereas the microtubules maintain cellular orientation to the stimulus gradient (3). It is only a subpopulation of monocytes within the entire circulating population of monocytes that has the ability to assume a polarized configuration following exposure to a chemoattractant (15).

Rheumatoid arthritis (RA) is a systemic disease (or group of disorders) in which chronic inflammation of synovial tissue causes the major clinical symptoms and is responsible for most of the associated chronic disability. Normal synovium is composed of a thin layer

of lining cells supported by fibrovascular connective tissue. The lining cells contain two subpopulations of cell types. Type A synoviocytes have characteristics of tissue macrophages including phagocytosis, and type B synoviocytes are secretory for synovial fluid (2). Synovium from affected joints in patients with RA is characterized by villous hypertrophy; lining cell proliferation; perivascular infiltration of the sublining connective tissue by monocyte/macrophages, plasma cells, activated lymphocytes, and PMN's; and infiltration of surrounding structures (17). The synovial proliferation and intense inflammation result in the progressive destruction of articular cartilage, subchondral bone, and tendons. The mechanism for this progressive destruction can be linked to the cell products of the inflammatory infiltrate (2,30). Synovial fibroblasts and synoviocytes taken from patients with RA have been shown to release large amounts of collagenase and prostaglandin E2 in vitro (18,19,20,21). Mononuclear inflammatory cells isolated from rheumatoid synovium produce a fibroblast activating/growth factor(s). Furthermore, these monocytes and lymphocytes are activated within the rheumatoid synovium, not in the peripheral blood (22). Thus, mononuclear cell signals may modulate all stages of joint disease in RA, from synovial hypertrophy to the

continued production of degradative enzymes and concomitant tissue destruction (22,30).

The mechanisms for synovial hypertrophy and joint damage in rheumatoid arthritis are becoming clearer, but the specific mechanisms for the recruitment of synovial inflammatory infiltrates in rheumatoid arthritis are unknown. Foreign agents, such as parvovirus or Epstein-Barr virus, have been implicated but the evidence is inconclusive (23,30). A genetically mediated altered control of cellular responses is likely, since there is a high association of rheumatoid arthritis with the specific histocompatibility (HLA) region of chromosome 6 known as HLA-DR4 (17,23,30).

We approached the question of recruitment of inflammatory infiltrates with the hypothesis that synovium from patients with rheumatoid arthritis produces a factor(s) that is a monocyte chemoattractant. To test this hypothesis, we cultured synovium from patients with rheumatoid arthritis, degenerative joint disease (DJD), or no history of joint disease (NL) and compared the ability to produce chemotactic activity for monocytes.

MATERIALS AND METHODS:

Cell Culture:

Synovial tissue was obtained from affected joints of patients with definite or classic rheumatoid arthritis, post trauma, or osteoarthritis at the time of arthroscopy or arthrotomy. Synovial tissue was also obtained from cadavers, within 12 hours of death, of patients with no known history of joint disease. After removal from the joint, synovial tissue is washed in Hank's balanced salt solution (HBSS, GIBCO). Synovial tissue is then aseptically minced and suspended in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% fetal calf serum (FCS, GIBCO). After several days there is outgrowth of adherent, fibroblast-like cells. Nonadherent tissue is discarded. This resulted in the establishment of homogeneous fibroblast-like synovial cell lines as described by other investigators (22,24,25). Five RA, 4 DJD, and 4 NL cell lines have been serially passaged and maintained in cell culture for up to six months. Cell lines are not always at identical passage number when media are conditioned for assays.

Preparation of Conditioned Medium:

Cultures of synoviocytes are grown to near confluence in DMEM and 10% FCS. Cultures are washed with four changes of buffered saline solution to remove residual serum. Synoviocytes are then incubated for 24 hours in serum-free medium containing carrier protein (50 ug/ml ovalbumin). After 24 hours, the conditioned medium is collected sterilely and centrifuged for 10 minutes at 3,000 x g to remove any cells that are present. Cell counts are done on flasks after conditioned medium is removed so that chemotactic activity can be determined per number of cells in culture.

Isolation of Monocytes and Polymorphonuclear Leukocytes:

Monocytes and PMN's are isolated from healthy human volunteers. Peripheral venous blood is mixed with acid-citrate-dextrose at a ratio of 4:1. Monocytes are obtained by first centrifuging the blood for 10 minutes to remove platelet rich plasma. After the plasma layer is aspirated, blood volume is restored with phosphate buffered saline, the blood is layered over Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, specific

gravity 1.077) and centrifuged for 30 minutes at 850 g. The mononuclear cell layer above the Ficoll-Hypaque is removed and washed twice in phosphate buffered saline. If present, red blood cells are lysed by a brief exposure to distilled water followed by a rapid restoration of isotonicity. Following washings, cells are resuspended in 3 ml of HBSS plus 2% bovine serum albumin (BSA, Sigma). Absolute cell counts are determined with a Coulter Counter.

PMN's are isolated from acid-citrate-dextrose anticoagulated blood by dextran sedimentation (28). Erythrocytes are lysed by a brief exposure to a hypotonic solution: the erythrocyte/PMN pellet is suspended in 7 ml of phosphate buffered saline (pH = 7.2), and twenty-one milliliters of H_2O are added followed by 7 ml of 3.6% NaCl 35 sec later (29).

Chemotaxis Assays:

Chemotaxis studies are performed in 48-well modified Boyden chambers purchased from Neuroprobe (Cabin John, M.D.), as previously described (26). For monocyte assays, leukocytes are suspended in HBSS plus 2% BSA with cells at a final concentration of 1.0×10^6 monocytes/ml. A polycarbonate filter with a 5.0 um pore

diameter and polyvinyl pyrrolidone coating is used. Cells are incubated in the assay chamber with the putative chemoattractant for 90 minutes at 37 degrees C. The filter is removed, cells are washed off the upper surface of the filter, and then remaining cells are fixed to the filter and stained with Diff-Quik (AHS del Caribe Inc., Aguado, Puerto Rico). Migration is measured as the number of cells that migrate completely across the filter per 400x high power field (HPF). A minimum of 5 fields per well are counted. All assay conditions are tested in duplicate except for the testing of column fractions which are not tested in duplicate, but are tested on multiple occasions.

For PMN studies, cells are suspended at a concentration of 2.5×10^6/ml; a nitrocellulose filter with a 5.0 um pore diameter is used; the suspension is incubated for 35 minutes; and migratory activity is read by the leading front method, i.e. the distance migrated by at least two cells (27). For both monocyte and PMN studies, each assay includes migration in response to HBSS plus 2% BSA as a negative control, and migration in response to the known chemoattractant N-formyl-methionyl-leucyl-phenylalanine (FMLP, Peninsula Labs, San Carlos, CA) at a concentration of 10^{-8} M as a positive control.

Chromatography:

Conditioned media from synoviocyte cultures are fractionated using either DEAE-HPLC ion exchange chromatography or Sephadex G100 column chromatography. For the former, 1.8 to 2.0 ml of serum free conditioned medium or buffer is loaded on a TSK DEAE 5PW ion exchange column (7.5 x 75 mm). The column is equilibrated with 20 mM Tris buffer, pH 7.5, and then eluted serially with 20 mM Tris buffer, pH 7.5, for 15 minutes followed by Tris buffer plus a linear sodium chloride gradient ranging from 0 to 0.7M. Twenty 1 ml fractions are collected at a flow rate of 1 ml/min. Fractions are dialyzed repeatedly against phosphate buffered saline using tubing with a molecular weight cutoff of 3,500.

For G100 column chromatography, a column with a bed volume of approximately 30 ml is packed with Sephadex G100 (Pharmacia, Piscataway, NJ), and washed extensively with phosphate buffered saline. The column is loaded with 200 ul of conditioned or non-conditioned media, or 5 ml of conditioned or non-conditioned media which had been dried using a Savant Speed Vac (Farmingdale, NY) and then reconstituted to 0.2 ml with phosphate buffered saline. The column is eluted with phosphate buffered saline, pH 7.2. Twenty-six 2 ml fractions are

recovered. Each 2 ml fraction is tested in chemotaxis assays at concentrations of 1%, 5%, and 25%. Size markers (in daltons) used for controls are vitamin B-12, 1,350; myoglobin (horse) 17,000; ovalbumin 44,000; gamma globulin (bovine) 158,000; and thyroglobulin 670,000 (Bio-Rad Industries, Richmond, CA).

Chemotactic Factor Characterization Assays:

Heat stability is tested by heating conditioned media to 100 degrees C for 10 minutes or 56 degrees C for 30 minutes along with controls. The resultant heat treated conditioned media are then tested in chemotaxis assays as described above.

Acid stability is tested by taking 1 ml of conditioned medium (approximate pH 7.6) and adding approximately 20 ul of 1M HCl solution to lower the pH to 3.0. The solution is then incubated for 30 minutes at room temperature. Approximately 20 ul of 1M NaOH solution is then added to raise the pH back to 7.6. Testing for pH is done with Hydrion Papers (Micro Essential Laboratory, Brooklyn, NY) and phenol red indicator. Medium is then tested in chemotaxis assays as described above.

Trypsin sensitivity of conditioned media are tested by incubating 5% conditioned media in HBSS plus 2% BSA with type IIIS bovine pancreas trypsin (Sigma) at a concentration of 10 mg/ml per sample tested for 90 minutes at 37 degrees C. Highly purified human C5a (generously supplied by Robert O. Webster, St. Louis University, St. Louis, MO) at a concentration of 10 ng/ml is used as a positive control. Following incubation, the trypsinized solutions are heat inactivated at 100 degrees C for 10 minutes. Chemotaxis assays are then performed as described above.

The effect of dialysis is evaluated by dialyzing conditioned media and controls against phosphate buffered saline through Spectrapor membrane tubing (Spectrum Medical Industries Inc., Los Angeles, CA) with a molecular weight cutoff of approximately 3,500. Dialysis retentates are then compared to non-dialyzed conditioned media and controls in chemotaxis assays as described above.

RESULTS:

As shown in Figure 1, supernatants from the 5 rheumatoid arthritis synoviocyte cell lines induced significantly more migration of monocytes than did supernatants from the 4 normal synoviocyte cell lines. In the studies depicted in Figure 1, media was obtained from near confluent cultures. The media had been conditioned for 24 hours without serum. Both conditioned and control media were tested at a 1% concentration in the lower well of the Boyden chamber. Migration induced by the chemotaxis assay buffer, HBSS plus 2% BSA, was defined as baseline (net migration of 0). Net migration was defined as the mean number of monocytes per high power field migrating through the filter in response to the putative stimulant, minus the average number of cells migrating in response to assay buffer. A 1% concentration of conditioned media from 5 different RA cell lines produced a mean net migration of 31.7 ± 8.3. In contrast, the mean net migration produced by 4 normal cell lines was 17.8 ± 5.3. Statistical comparisons were performed by the student's T-test. The difference between RA and NL was highly significant ($p<0.01$). In simultaneous assays, the control chemoattractant, FMLP at an optimal concentration (10^{-8}M), induced a net average migration

of 27.9 ± 2.2, slightly less than RA culture. Conditioned media supernatants from DJD cultures were slightly less active than RA cultures, inducing a mean net migration of 28.3 ± 8.4 and significantly more active than supernatants from normal cell lines ($p<0.05$). Non-conditioned media alone induced a net migration of 10.9 ± 2.3. If the net migration is averaged to the total number of cells in each culture at the time of media conditioning, the difference in net migration between RA and NL is less significant ($p<0.2$). The same holds true for the difference in net migration between DJD and NL ($p<0.2$). Thus, the differences in net migration produced by RA, DJD, and NL cell lines may be partially explained by differences in cell growth rates (i.e. metabolic activity).

As described earlier, leukocyte migration can be classified as chemotactic (stimulated directed migration) or chemokinetic (stimulated random migration). Chemotaxis, in contrast to chemokinesis, is optimal with a stimulus gradient. To determine if the migration induced by synoviocyte cell cultures was chemotactic or chemokinetic, checkerboard assays (conditioned medium above and/or below the filter) were performed. The migration induced by RA cell cultures was shown to be chemotactic by checkerboard analysis in 4 out of 5 RA cultures. The representative checkerboard

assay depicted in Table 1 indicates that maximal migration was produced when conditioned medium was only below the filter, i.e. the situation when a maximal concentration gradient is present. By checkerboard analysis this RA conditioned medium is definitely chemotactic, although it also has some chemokinetic effect (like most chemoattractants), i.e. it stimulated some increased migration when conditioned medium are both above and below the filter. The checkerboard assays were run at conditioned media concentrations of 1% and 5% with controls of HBSS plus 2% BSA, non-conditioned medium, and FMLP.

In contrast to RA cell lines, in checkerboard assays of all 4 normal cell lines, migration is not increased in the presence of a stimulus gradient (conditioned media only below the filter). A representative checkerboard assay is shown in Table 2. Instead, migration is maximal with conditioned medium above the filter or both above and below the filter. This is indicative of chemokinesis, not chemotaxis. Checkerboard assays of DJD cell lines showed that migration was generally chemokinetic (3 out of 4 cell lines), not chemotactic (1 out of 4 cell lines).

Some chemoattractants exhibit a preferential effect on either monocytes or PMN's (6). As seen in Table 3, conditioned media had only a minimal ability to induce

migration of PMN's. Net migration was defined as the induced distance migrated (in um/35 min) with the sample tested, minus the mean induced distance migrated by the negative control (HBSS plus 2% BSA). Only a single RA cell line and a single DJD cell line were tested, and although minimal migration above baseline was induced, there was no significantly greater migration than that induced by non-conditioned medium (MEM). At 5% concentrations a slight inhibatory affect was actually seen as compared to non-conditioned medium. In contrast, the positive control (FMLP) induced marked migration above baseline.

The nature of the chemoattractant produced by the RA cell lines was further characterized in multiple studies shown in Table 4. The chemoattractant is heat stable with no loss of activity after heating to 100 degrees C for 10 minutes or 56 degrees C for 30 minutes. The chemoattractant is also acid stable with minimal loss of activity following incubation in a solution of HBSS plus 2% BSA at pH 3.0 for 30 minutes. In contrast, the chemoattractant was highly trypsin sensitive with a loss of 95% of its activity following incubation with trypsin solution for 90 minutes at 37 degrees C. Human C5a, a known monocyte chemoattractant (4) which is inactivated by trypsin, was used as a control. The trypsin sensitivity of the chemoattractant is indicative

of a protein. After determining that the chemoattractant is heat stable, it was possible to use the simpler technique of a trypsin sensitivity assay which employs heat inactivation rather than the more complex assays requiring agarose bead bound trypsin. Dialysis against phosphate buffered saline using membrane tubing with a molecular weight cutoff of approximately 3,500 resulted in no loss of chemotactic activity.

The nature of the chemoattractant was further characterized by HPLC ion exchange chromatography in preliminary studies (Figure 2). Cultured supernatants or running buffer were chromatographed on a DEAE HPLC TSK 5PW (7.5 x 75 mm) ion exchange column. The column was equilibrated and eluted serially with 20 mM Tris buffer, pH 7.5 followed by Tris buffer plus a linear sodium chloride gradient ranging from 0 to 0.7M. Twenty separate column fractions were obtained. Fractions were tested at a 5% concentration (volume per volume) in the lower well of the Boyden chamber in 3 or 4 independent assays. Using supernatants from the culture of a representative RA synoviocyte cell line fraction 14 (which contains anionic substances) induced significantly more migration ($p<0.05$, student's T-test) than comparable fractions from either DJD or buffer.

Gel filtration chromatography was performed with a Sephadex G100 column (Figure 3). Twenty column fractions were obtained and tested at a concentration of 25% (if media were not concentrated prior to chromatography) or 5% (if media were concentrated prior to chromatography) in the lower wells of Boyden chambers. In 5 independent assays, fractions 3 and 7 from culture supernatants of a representative RA synoviocyte cell line induced significantly more migratory activity ($p<0.05$) than comparable fractions from the control medium. Fractions 4, 5, and 6 from the RA culture supernatant induced greater migration that was not statistically significant. The elution profile of size markers indicated an M_R between 158,000 and 670,000 dalton. The possible presence of lower molecular weight chemotactic factors was difficult to determine due to the high background activity in the control.

DISCUSSION:

Rheumatoid arthritis affects an estimated 1% of the world's population (30). The etiology of rheumatoid arthritis is presently unknown and current therapy frequently is neither curative nor satisfactory in stopping its progression. The primary manifestation of rheumatoid arthritis is synovitis, characterized by inflammatory infiltrates of monocytes, lymphocytes, plasma cells, and PMN's. The synovium proliferates with chronic inflammation, eventually infiltrating surrounding structures with irreversible destruction of articular cartilage, subchondral bone, and tendons. The underlying mechanisms for the initial recruitment of inflammatory infiltrates into synovium is unknown.

We hypothesized that rheumatoid synovium itself may produce factors which are chemoattractants for monocytes and may initiate the inflammatory response. Our in vitro studies support this hypothesis; we have shown that cultured rheumatoid synoviocytes produce a factor which is chemotactic for monocytes in Boyden chamber assays, whereas cultured normal synoviocytes do not. Characterization studies indicate that this factor(s) is a large molecular weight, anionic protein which is heat and acid stable, but trypsin sensitive.

The elucidation of a monocyte chemoattractant produced by rheumatoid synovium raises several questions. First, is this in vitro finding representative of actual in vivo events? Boyden chamber assays have been used as accurate diagnostic tools for monocyte and PMN locomotive dysfunction diseases (26). It is reasonable to assume that soluble factors that act as chemoattractants in Boyden chamber assays do in fact have the properties of chemotactic factors. However, the concentrations that conditioned media supernatants are tested at may or may not correlate to the actual in vivo levels of the factors. In addition, chemoattractants may be chemotactic at low concentrations but act as inhibitory agents at higher concentrations (4,27). In all of our checkerboard assays (testing if migration was chemotactic) conditioned supernatants were tested in concentrations of 1% and 5%, and occasionally at 25%. Dose response migration was evident in some, but not all assays. This may indicate that we were testing near plateau levels, i.e. levels that already induced maximum migration. Further studies to determine the lowest concentration that induces migration would be useful. Migration was quantified with a known powerful chemoattractant, FMLP, as a positive control. The net migration induced by RA synoviocytes was consistently equal or greater (though

not at statistically significant levels) than that induced by FMLP. This indicates that the chemotactic property of the synovial factor(s) is fairly powerful.

The tissue culture techniques used were standard for those routinely used in culturing synovial fibroblasts (22,24,25). Inherent in this process is the selection of the faster growing subpopulations of cells, to eventually form a morphologically homogeneous population. Cells which may be important to the disease processes through secreting soluble factors, having important cell-to-cell interactions, or providing an important cellular matrix are potentially lost. However, rheumatoid synovial fibroblasts similar to those which we have used in our studies have several qualities which differentiate them from "normal" synovial fibroblasts. These include higher growth rates, increased rates of glucose utilization, lactate production, and production of lysosomal enzymes (25,40,41). These differences between rheumatoid cell lines and normal cell lines persist through multiple passages, implying a permanent alteration in cell function. Studies are currently being done to see if the cell lines, which have now been in tissue culture for almost a year, still produce conditioned medium with the same characteristics that they had 6-9 months ago.

Additionally, controlling for cell culture passage number should be done.

The characterization studies were carried out primarily on a single RA cell line which consistently produced chemaoattractant activity. These initial studies indicate that the chemoattractant is a protein. The trypsin sensitivity is the most convincing evidence for this. However, trypsin may have a toxic effect on the chemoattractant as opposed to a proteolytic effect. Controls for a possible toxic effect were not run (other than assay buffer), and this would be a worthwhile experiment to do. The HPLC anion exchange data is also consistent with a protein identity, with the chemoattractant eluting out in an anionic fraction. Another possible study to furnish evidence whether the chemotactic factor is a protein would be to test the effect of cyclohexamide (a protein synthesis inhibitor) on the production of chemoattractant. Multiple assays on conditioned media from single representative cell lines run through an HPLC column resulted in consistent statistically significant findings of a single fraction on ion exchange and two fractions on gel filtration which induced migration. The migration recovered from column chromatography, however, was not maximal compared to pre-HPLC levels. This may have been due to one or more chemoattractants "sticking" on the column, causing

a loss of overall activity. The results from the anion exchange column, as well as the HPLC gel filtration studies must only be considered preliminary; this data is from a single run of conditioned media and controls. In order to help characterize the chemoattractant, HPLC data should be generated on more than single representative cell lines and on more than a single run.

With the HPLC data as preliminary, it is difficult to speculate as to the identity of the chemoattractant. One possibility might be collagen. Collagen and collagen degradation products are known monocyte chemoattractants with a wide range of molecular weights (8), and are produced by fibroblasts. Collagen degradation products are found at sites of tissue injury and inflammation, and it is certainly possible that they act as perpetuators in monocyte chemotaxis even if they did not produce the initial chemotactic activity. In addition, collagen degradation products are not chemotactic for PMN's (8), which is consistent with our results. Further studies should help determine whether or not collagen degradation products are being produced by rheumatoid synoviocytes. Two other possibilities are interleukin-1 and fibronectin (a large molecular weight protein). Macrophages have been shown to produce fibronectin, which is chemotactic for fibroblasts (44). Fibronectin, however, is heat labile (44) while the

chemotactic factor we are studying is heat stable.

Cultured human fibroblasts (dermal) have previously been shown to produce leukocyte chemoattractants (11). Characterization studies of these chemoattractants showed heat stability, trypsin sensitivity, and molecular weight greater than 100,000 dalton. All of these are consistent with our findings for cultured human synovial fibroblasts. However, the chemoattractant(s) we are studying have thus far been chemotactic for monocytes but not PMN's, while the dermal fibroblast produced chemoattractants are chemotactic for both monocytes and PMN's. This would indicate that the two different types of fibroblasts are secreting chemically distinct factors.

Although undoubtedly a large number of factors are involved in the pathogenesis of rheumatoid arthritis; the demonstration of the production of a chemoattractant by rheumatoid synovium could contribute significantly to the understanding of the pathogenesis of this disease. This provides a potential mechanism for initial and maintained recruitment of chronic inflammatory infiltrates within rheumatoid synovium. Again, the question is raised, why are rheumatoid synoviocytes secreting a chemotactic factor, while normal synoviocytes do not? There are several possible explanations. Rheumatoid synovium may be fundamentally

different than normal synovium on a genetic basis (given the high incidence of HLA-DR4 in RA), and may secrete a unique soluble factor. Rheumatoid synovium may produce a larger than normal amount of a common cell product that is only chemotactic in quantities produced by rheumatoid synovium. To test this possibility, normal synovium could be stimulated to grow with growth factors or bacterial products in vitro and then checked for the production of chemoattractants. Rheumatoid synovium may be hyperplastic due to autocrine functions such as production of a unique growth factor or overproduction of a normal growth factor, and this growth factor may act as a chemoattractant for monocytes. Known growth factors such as platelet derived growth factor (PDGF) have been shown to be chemotactic for monocytes (5). Alternatively, growth factors could induce synovial fibroblast proliferation (42,43) and the products of proliferation such as collagen or elastin could induce chemotaxis (7,8). Infectious agents could initiate an inflammatory response (23,30) with a subsequent cross reactivity to synovium, and a specific genetic haplotype may increase the possibility of cross reactivity. Patients with rheumatoid arthritis may have an underlying abnormality in their monocytes/macrophages which cause increased reactivity to their own synovium. This would not explain our results of monocytes from

normal patients reacting with rheumatoid synovium but not normal synovium, unless abnormal monocytes stimulate synovial proliferation which then induces inflammatory infiltrates. There is some evidence for this possibility. Recent reports have shown that activated monocytes produce a PDGF-like factor which can stimulate fibroblast proliferation (36).

Once the mononuclear inflammatory cells are in the synovium a myriad number of actions have been shown to occur. Mononuclear cells isolated from rheumatoid synovium have been shown to be activated in situ in the synovium, and these activated leukocytes produce fibroblast activating factors (22). Mononuclear leukocytes have been shown to produce factors which induce considerable response in rheumatoid synovium. The monocyte/macrophage product interleukin-1 and the t-lymphocyte product immune interferon, as well as other leukocyte derived proteins, stimulate synovial fibroblast proliferation (21,24,37,38,39). Synovial tissue has also been shown to produce interleukin-1 (45), however, in those studies it is not clear whether or not the synovium was free of monocyte produced interleukin-1 prior to assaying. Interleukin-1 has also been shown to mediate increased production of prostaglandin E_2, collagenases, and plasminogen activator, all of which can be linked to increased

cartilage destruction and enhanced inflammatory response (18,19,20,21,31,32,33). In addition, rheumatoid synovium has been shown to have increased pinocytotic activity, inducible morphological changes, and enhanced neutral protease activity when stimulated (32,34,35). The normal inflammatory response to acute injury has monocytes regulating the functions of other proliferating cells (4). In rheumatoid synovium, the multiple positive feedback mechanisms of inflammatory cell products and proliferating synovial fibroblast products seem to go unchecked, stimulated beyond normal response to become destructive to the host.

SUMMARY:

Rheumatoid arthritis is a systemic disease characterized by the destructive proliferation of joint tissue (synovium). Rheumatoid synovium is characterized by perivascular infiltration of the sublining connective tissue by monocyte/macrophages, plasma cells, lymphocytes, and polymorphonuclear leukocytes, as well as villous hypertrophy, lining cell proliferation, and infiltration of surrounding structures. Monocyte/macrophages in the synovium of patients with rheumatoid arthritis actively contribute to joint disease, but the mechanisms for their recruitment are unknown. We hypothesized that rheumatoid synovium produces a chemoattractant for monocytes. To test this hypothesis, we established synoviocyte cultures from 5 patients with rheumatoid arthritis, 4 with degenerative joint disease, and 4 without significant joint disease. In modified Boyden chamber assays we showed that synoviocytes from patients with rheumatoid arthritis secrete a factor(s) which is chemotactic for monocytes. The chemotactic factor is heat and acid stable, but trypsin sensitive. In contrast to synoviocytes from patients with rheumatoid arthritis, synoviocytes from individuals without joint disease do not appear to secrete chemotactic substances. In preliminary studies,

column chromatography suggests that the chemotactic factor from rheumatoid synoviocytes is anionic and has a molecular weight between 158,000 and 670,000 dalton. The secretion of chemotactic activity by synoviocytes could contribute significantly to the pathogenesis of rheumatoid arthritis.

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Table 1.

Checkerboard Analysis of Migration Induced by Culture Supernatant from RA Synoviocytes Demonstrating Chemotaxis

		% Supernatant Above Filter		
		0	1	5
% Supernatant Below Filter	0	32.9±3.0	45.6±3.7	43.3±2.8
	1	69.8±3.5	57.3±2.4	not done
	5	67.4±1.9	not done	46.5±3.6

Table 1. Data are given as the mean number of monocytes/high power field ± SD migrating through the filter. Greatest migration in the presence of a gradient (supernatant only below the filter) is indicative of chemotaxis. Data are from a representative assay with other assays also showing a consistent chemotactic effect.

Table 2.

Checkerboard Analysis of Migration Induced by Culture Supernatant from NL Synoviocytes Demonstrating Lack of Chemotaxis

		% Supernatant Above Filter		
		0	1	5
% Supernatant Below Filter	0	21.8±1.5	29.5±3.9	28.3±2.7
	1	28.8±2.3	28.6±2.7	not done
	5	28.7±2.7	not done	31.1±2.8

Table 2. Data are given as the mean number of monocytes/high power field ± SD migrating through the filter. Migration is not increased in the presence of a gradient (supernatant only below the filter). This is indicative of chemotaxis not being present. Data are from a representative assay with other assays also consistently showing no chemotactic effect.

Table 3.

Demonstration of Lack of Chemotactic Activity for Polymorphonuclear Leukocytes Produced by Conditioned Media From RA and DJD Synoviocytes

Sample tested	Concentration	Net PMN Migration
RA	5%	3
DJD	5%	6
MEM (control)	5%	9
FMLP (control)	10^{-8}	53
RA	25%	15
DJD	25%	15
MEM (control)	25%	12
FMLP (control)	10^{-9}	73

Table 3. Conditioned media from single representative RA and DJD cell lines at concentrations of 5% and 25% (vol/vol) induced no significant migration as compared to non-conditioned medium (MEM) or FMLP (positive control). Net migration is defined as induced migration (in um/35min), minus migration induced by assay control (HBSS plus 2% BSA).

Table 4.

Chemotactic Activity is Heat and Acid Stable and Trypsin Sensitive

Treatment	% Change from Control
Heating:	
100°C x 10 minutes	0%
56°C x 30 minutes	+11%
Acid:	
pH 3.0 x 30 minutes	-6%
Trypsin	-95%
Dialysis (3500 MW cutoff)	+12%

Table 4. Conditioned medium from a representative RA cell line had chemotactic activity that was characterized by heat and acid stability, trypsin sensitivity, and dialysis stability at a molecular weight cutoff of 3,500. Control was conditioned medium from the same RA cell line without treatment.

Figure 1.

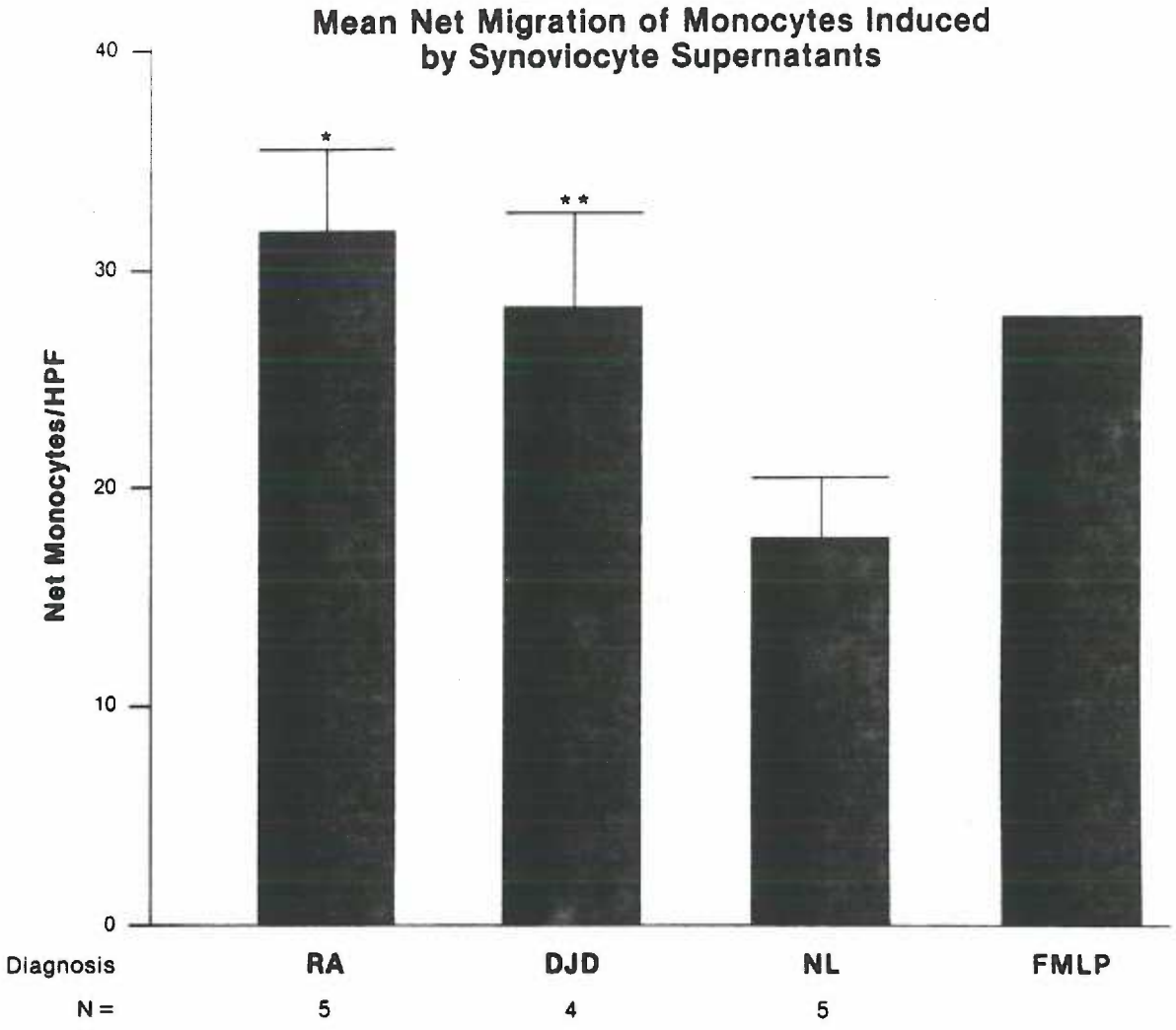

Figure 1. A 1% concentration of conditioned medium from 5 different RA cell lines produced a mean net migration of 31.7 $\pm$ 8.3. In contrast , the mean net migration induced by 4 NL cell lines was 17.8 $\pm$ 5.3. The difference between RA and NL was significant ($p<0.01$). The difference between DJD (mean net migration of 4 cell lines, 28.3 $\pm$ 8.4) and NL was also significant ($p<0.05$). The mean net migration induced by FMLP (positive control) was 27.9 $\pm$ 2.2. Mean net migration was defined as the average number of monocytes migrating through the filter in response to stimulant minus the average number of cells migrating in response to assay buffer.

Figure 2.

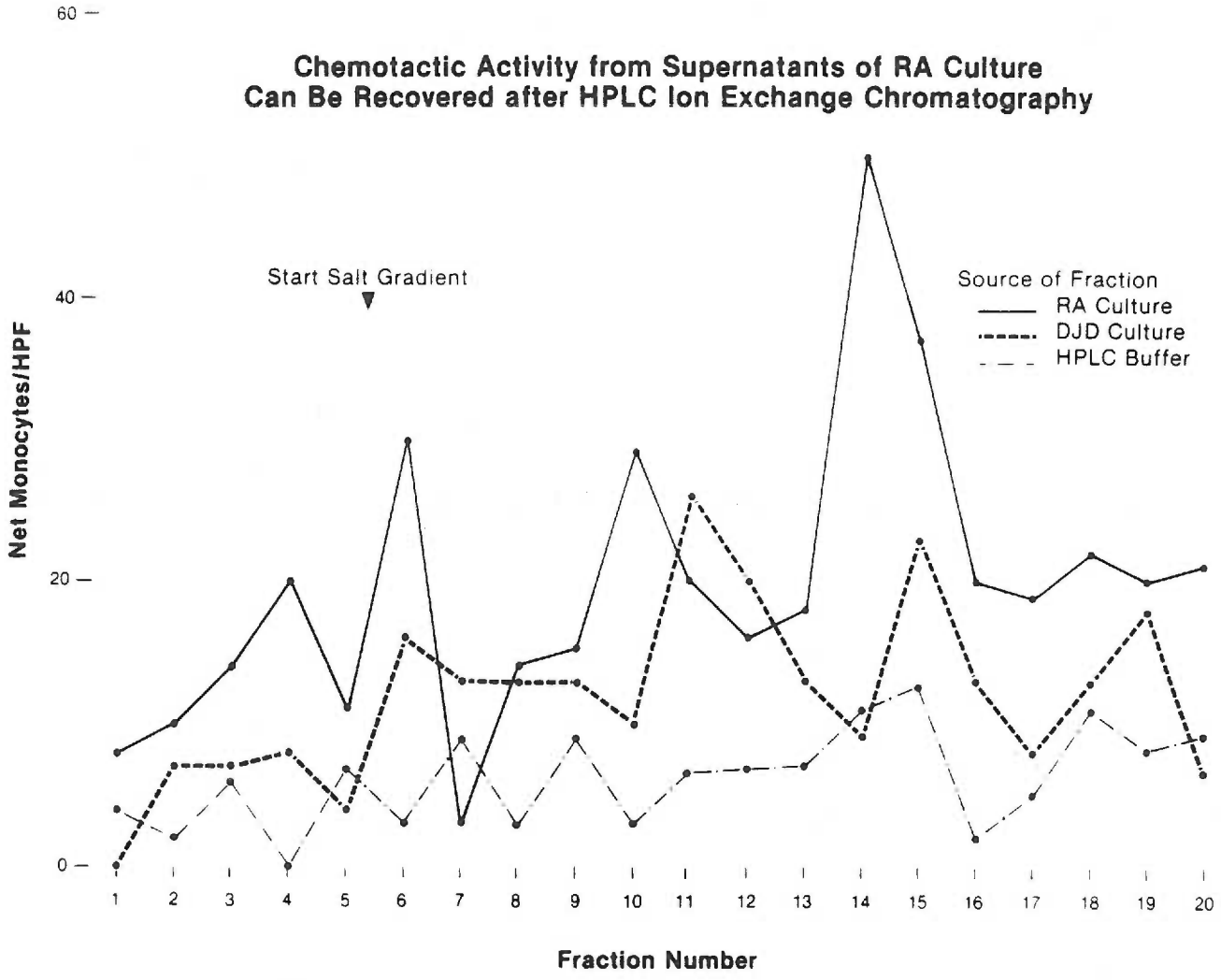

Figure 2. Cultured supernatant or running buffer were chromatographed on an HPLC ion exchange column. Twenty separate column fractions were obtained. Fractions were tested at a 5% (vol/vol) concentration in the lower well of Boyden chambers in 3 or 4 independent assays. Fraction 14 (which contains anionic substances) from conditioned media of a representative RA line induced significantly more migration ($p<0.05$) than comparable fractions from either DJD or buffer.

Figure 3.

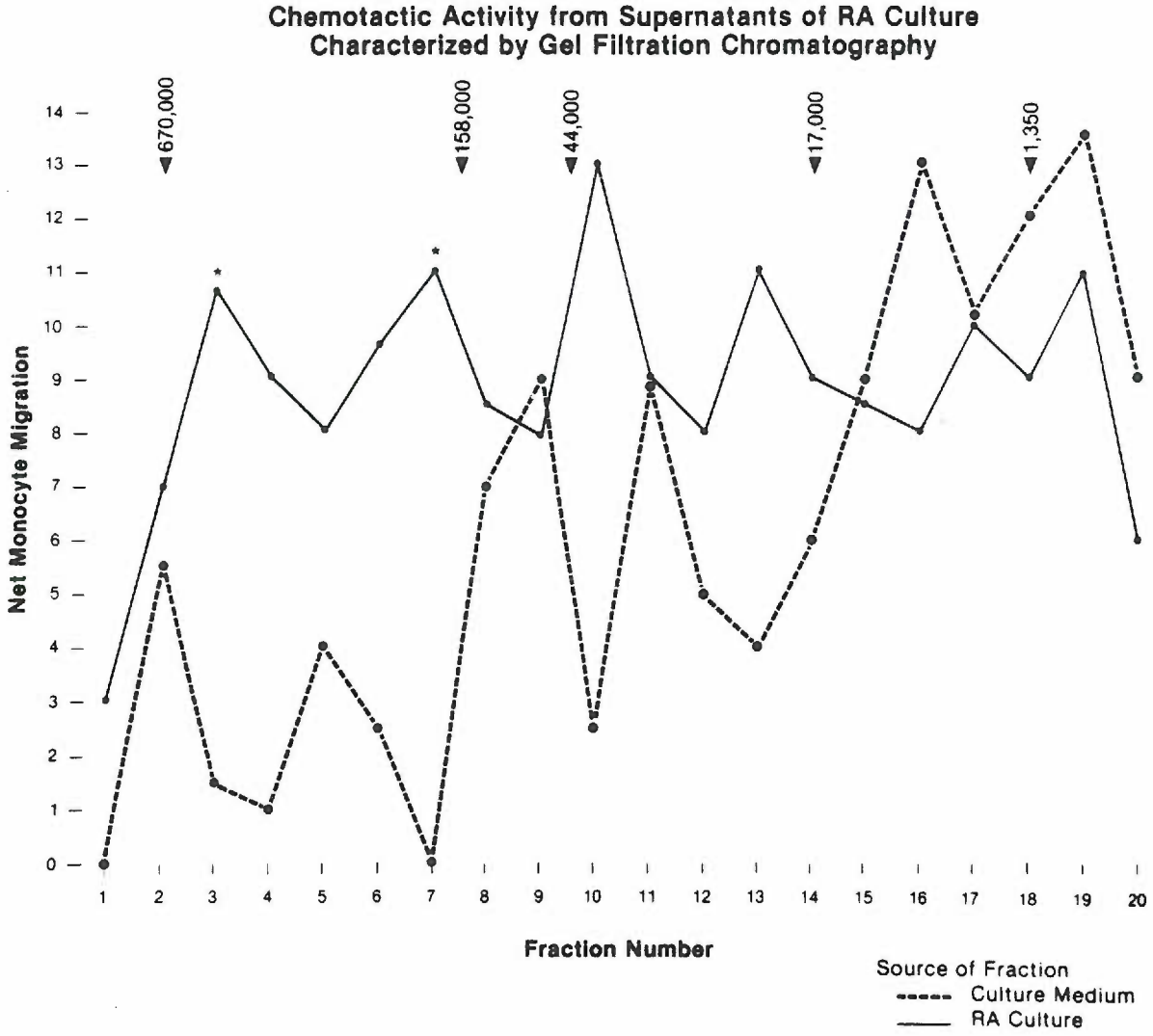

Figure 3. Gel filtration chromatography was performed with a Sephadex G100 column. Twenty separate fractions were obtained and tested at either 5% or 25% concentrations (depending whether or not media was concentrated prior to chromatography) in the lower wells of Boyden chambers. In 5 independent assays, fractions 3 and 7 from culture supernantants of a representative RA synoviocyte cell line induced significantly more migratory activity ($p<0.05$) than comparable fractions from the control medium. The elution profile of size markers indicates an M_R between 158,000 and 670,000 dalton.