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A chemotactic inhibitor in synovial fluid

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Summary. Synovial fluid was found to contain an inhibitor of neutrophil chemotaxis. The activity of this inhibitor was masked in native synovial fluid, but could be detected in fluid in which complement had been deactivated by mild heating. The inhibitor was most effective against the chemotactic activity of zymosan-activated serum ($C5a_{des\ arg}$). It had little effect when N-formyl-methionyl-leucyl-phenylalanine served as chemoattractant. Inhibition was not the result of a direct effect on the neutrophils, since incubation of cells with synovial fluid did not alter their chemotactic response. The inhibitory activity was destroyed by boiling the synovial fluid or treating it with trypsin, suggesting that it is a protein (or proteins); it was not affected by hyaluronidase treatment. Gel filtration revealed that the inhibitor was present in native as well as decomplemented synovial fluid, and that its molecular weight was in the vicinity of 25,000. It is proposed that this inhibitory activity plays a role in the regulation of the inflammatory response in joints.

INTRODUCTION

It is becoming increasingly evident that neutrophil migration into an inflammatory site is a self-amplifying phenomenon. The first few neutrophils to arrive at a site of inflammation are probably attracted there by chemotactic factors which are released by the noxious agent (e.g., invading microorganisms) responsible for initiating the inflammatory response. Once there, however, these neutrophils will be provoked into discharging their specific granules and initiating $O_2^-\cdot$ production by high local concentrations of the same chemotactic factors which at lower concentrations attracted them initially (Weissmann, Smolen & Korchak, 1980). It has been shown that specific granule contents contain a protease that cleaves complement component C5 to release the powerfully chemotactic fragment, C5a (Wright & Gallin, 1977; Ward & Hill, 1970; Goldstein & Weissmann, 1974). In addition, $O_2^-\cdot$ acts on a component of serum to produce an as yet uncharacterized chemotactic lipid (Petrone *et al.*, 1980; Perez, Weksler & Goldstein, 1980). Chemotactic factors will therefore be released by the first few neutrophils at an inflammatory site, attracting more neutrophils, which in turn will release still more chemotactic factors, attracting yet more neutrophils. Thus, the inflammatory response has an explosive character which, if uncontrolled, could lead to serious consequences. This suggests that there must be a mechanism to regulate the inflammatory response so as to prevent its development at inappropriate times and places.

Because the self-amplifying character of an inflammatory reaction appears to be mediated in part through chemotactic factors, we reasoned that a chemotactic inhibitor might serve as a component of this postulated regulatory mechanism. We therefore

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sought such an inhibitor in knee-joint synovial fluid. We chose this fluid as a potential source of the inhibitor for two reasons: first, because the knee is constantly subjected to minor trauma, yet does not become inflamed in response to this trauma, suggesting that it may possess an anti-inflammatory regulatory system; and second, because quasi-normal synovial fluid is easily obtained from the knees of patients with degenerative joint disease. In this paper, we report the presence of a low level chemotactic inhibitor in knee joint synovial fluid, and postulate a role for this inhibitor in the regulation of inflammation in joints.

MATERIALS AND METHODS

Ficoll-Paque, Dextran 70 (Macrodex 6% in 0·9% NaCl) and Sephadex G-150 were obtained from Pharmacia, Piscataway, N.J. Zymosan, hyaluronidase (bovine testes, type VI-5), bovine serum albumin (Type V), N-formyl-methionyl-leucyl-phenylalanine (f-met-leu-phe), trypsin (porcine pancreas, type IX) and soybean trypsin inhibitor (Type I-s), were purchased from Sigma, St. Louis. Gey's balanced salts solution was obtained from Microbiological Associates, Walkersville, MD. Other reagents were the best grade commercially available, and were used without further purification. Modified Boyden and blind well chambers were purchased from Nucleopore Corp., Bethesda, MD, and corresponding filters (Membranafilter, 3 μm pore size) from Sartorius, Göttingen, W. Germany.

Neutrophils

Neutrophils were prepared by dextran sedimentation followed by centrifugation over Ficoll-Hypaque, as described elsewhere (Crowley *et al.*, 1980). The cells (> 99% granulocytes) were suspended at 10^6 cells/ml in Gey's solution containing 6 mg/ml bovine serum albumin.

Synovial fluid

Knee-joint synovial fluid was obtained from patients with osteoarthritis. The fluid was centrifuged at 1500 *g* for 10 min at 4° to remove cells and debris, then divided into aliquots and stored at −70° until use. Complement was inactivated by heating the fluid for 30 min at 56° just before use.

$C5a_{des\ arg}$

$C5a_{des\ arg}$ was prepared from normal human serum according to the method of Fernandez & Hugli (1976). The material was pure by gel electrophoresis (14% Laemmli gel [Laemmli, 1970], silver stain [Oakley, Kirsch & Norris, 1980]) and had the chemotactic properties reported by Fernandez *et al.* (1978).

Chemotaxis

Chemotaxis was assayed by the leading front technique (Zigmond & Hirsch, 1973), using zymosan-activated serum or f-met-leu-phe as chemotactic factor. Zymosan-activated serum was prepared by incubating normal human serum with zymosan (10 mg/ml) for 20 min at 37°. After removal of the zymosan by centrifugation (800 *g* for 10 min at 4°), the serum was divided into aliquots and stored at −70° until use. In the assays, the neutrophil suspension was placed in the upper compartment of the apparatus (Boyden chamber or blind well chamber), and chemoattractant (Gey's solution containing the desired quantity of chemotactic factor, synovial fluid as indicated, and bovine serum albumin at a final concentration of 6 mg/ml) in the lower compartment. Incubations were carried out at 37° for 45 min. Random migration was determined by measuring migration against Gey's solution containing 6 mg/ml bovine serum albumin with and without synovial fluid; the synovial fluid was found to have no effect on random migration.

Gel filtration of synovial fluid

Synovial fluid (2 ml) was treated for 10 min at 37° with 5 units/ml of hyaluronidase, to reduce its viscosity prior to gel filtration. Where indicated, the hyaluronidase-treated fluid was decomplemented as described above. It was then centrifuged at 1500 *g* for 10 min at 4°, and the supernatant was applied to a 1·6 × 25 cm column of Sephadex G-150 which had previously been calibrated with chymotrypsinogen A (mol. wt. 25,000), ovalbumin (mol. wt. 43,000) and aldolase (mol. wt. 153,000), then equilibrated with Gey's solution. The column was eluted with the same buffer, collecting 2 ml fractions. The entire procedure was carried out at 4°.

Each fraction was assayed for protein (A_{280}) and for chemotactic inhibitory activity. Chemotactic assays were performed in blind well chambers, using as chemoattractant 1% zymosan-activated serum plus bovine serum albumin (6 mg/ml) in the fraction to be tested.

Calculations

Chemotaxis was calculated as the distance travelled in

Table 1. The effect of synovial fluid on the chemotaxis of neutrophils toward zymosan-activated serum

	Inhibition of chemotaxis (%)	
Serum concentration (%)	Native fluid	Decomplemented fluid
10	−5·7±5·2	21·7±8·7
3	11·5±9·1	53·4±8·3*
1	−1·3±9·2	59·1±3·4*
0·3	−22·4±11·6	63·7±5·4*

* Different from 0, $P<0{\cdot}001$.

Chemotaxis was measured in modified Boyden chambers as described in 'Materials and Methods'. Synovial fluid was added to the chemoattractant in a concentration of 10% (v/v). For each assay carried out with synovial fluid in the chemoattractant, a control assay was carried out under conditions which were identical in every respect except that the synovial fluid was replaced by an equal volume of Gey's solution. In the experiment with native synovial fluid, values for control chemotaxis (corrected for random migration of 55·3±6·8 μm) were 73·0±6·0, 77·7±6·1, 66·7±5·8 and 51·3±9·2 μm, respectively, for 10%, 3%, 1% and 0·3% zymosan-activated serum. With decomplemented synovial fluid, these values (corrected for random migration of 47·0±2·9 μm) were 59·0±4·2, 67·3±5·1, 63·0±6·6 and 53·5±6·0 μm, respectively. The results are expressed as the mean±SE of six experiments, each using a different synovial fluid specimen and neutrophils from a different donor.

response to chemotactic factor minus the random migration. Inhibition of chemotaxis was calculated as follows: percent inhibition = 100 × [1 − (chemotaxis with synovial fluid/chemotaxis without synovial fluid)]. The significance of differences was determined by Student's *t* test.

RESULTS

A chemotactic inhibitor in synovial fluid

A low-level inhibitor of chemotaxis was initially sought by examining whether synovial fluid could neutralize the chemotactic activity of zymosan-activated serum. The first experiments failed to disclose such an inhibitor, even when very low concentrations of chemoattractant were used in the assay (Table 1, 'Native fluid'). Previous studies showing that complement components are present in synovial fluid, however, raised the possibility that the hypothetical inhibitor might be swamped by chemotactic factors released from synovial fluid complement during the course of the assay. This possibility was strengthened by the finding that chemotactic activity is generated in native synovial fluid, but not in synovial fluid that had been decomplemented by heating at 56° for 30 min, when the fluid is exposed to zymosan (Table 2). The original experiments were therefore repeated using decomplemented synovial fluid. Under these conditions, it was possible to show that this fluid did in fact contain the postulated chemotactic inhibitor (Table 1, 'Decomplemented fluid'). There appeared to be no effect of the fluid on the random migration of the cells (Table 2, rows 2 and 3).

The results presented in Tables 1 and 2 were obtained using the leading front method, a technique which can be criticized on the grounds that chemotaxis of an entire neutrophil population is inferred from measurements made on 10 cells or fewer. To validate these results, we reassessed chemotaxis into the filters from the 'decomplemented fluid' experiments (Table 1) using a method in which cell numbers were determined as a function of distance from the upper surface of the filter (Maderazo & Woronick, 1978). These results (Fig. 1) were in agreement with those obtained by the leading front method. We therefore concluded that the leading front method was satisfactory for our purposes, and employed this method in the rest of our studies.

Table 2. Chemotactic factor activity in synovial fluid

Chemoattractant	Chemotaxis (μm)
Native synovial fluid, activated (7)	45·6±4·4
Native synovial fluid, untreated (3)	3·7±1·8
Decomplemented synovial fluid, activated (6)	6·0±1·1
Serum, activated (7)	63·3±5·8

Chemotaxis was measured in modified Boyden chambers as described in 'Materials and Methods', using the chemoattractants indicated at concentrations of 10% (v/v) in Gey's solution. Activation of synovial fluid by zymosan was carried out by the procedure described in 'Materials and Methods' for the activation of serum. Values for chemotaxis are corrected for random migration (43·8±6·5 μm). The number of experiments with each chemoattractant is given by the figure in parentheses. Each experiment was carried out using a different synovial fluid specimen and neutrophils from a different donor. Results are expressed as the mean±1 SE.

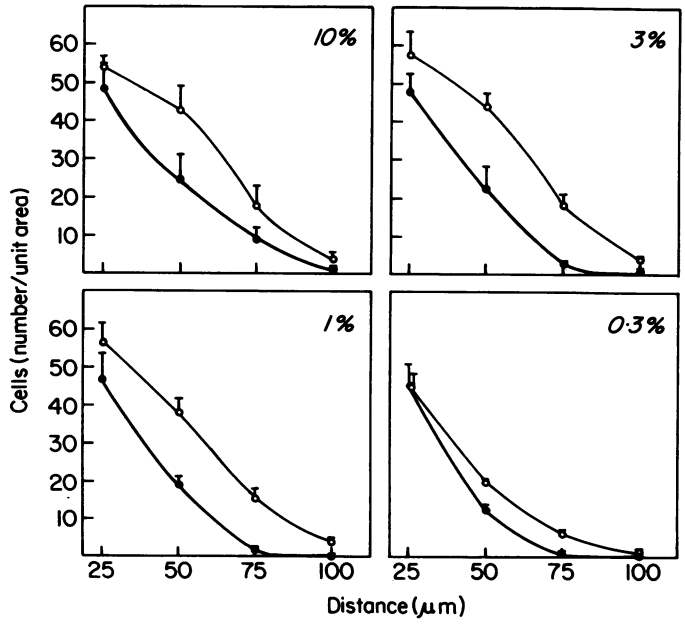

Figure 1. Validation of the leading front assay method. Filters from the 'decomplemented fluid' experiments reported in Table 1 were examined under the microscope at magnification of ×400. Cells in focus at 25 μm intervals from the upper surface of the filter were counted with the aid of a net micrometer (Carl Zeiss, Inc.). Each point represents the mean±1 SE of numbers of cells from each of six filters; each number was the average of the number of cells enclosed by the micrometer grid in each of two fields at each distance. The values are corrected for random migration of 9·1±2·5 and 1·3±0·14 cells, respectively, for 25 and 50 μm. (○) serum alone; (●) synovial fluid added.

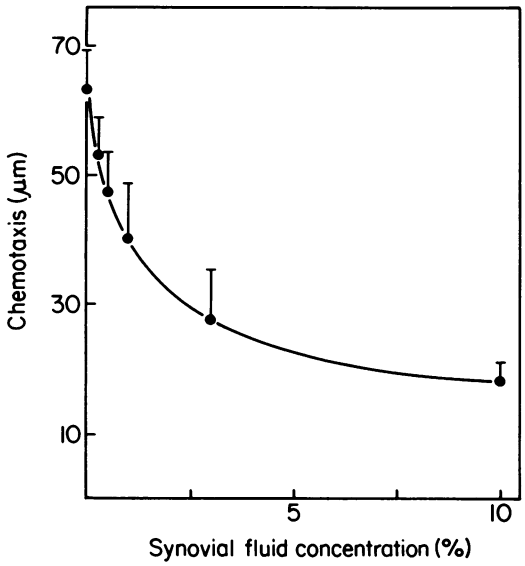

Figure 2. Inhibition of neutrophil chemotaxis as a function of synovial fluid concentration. Chemotaxis was measured in Boyden chambers as described in 'Materials and Methods'. The chemoattractant was 0·5% zymosan-activated serum in Gey's solution containing decomplemented synovial fluid at the concentrations indicated. The values for chemotaxis were corrected for random migration of 57·7 ± 6·4 μm. The results are expressed as the mean ± 1 SE of three experiments, each using a different synovial fluid specimen and neutrophils from a different donor.

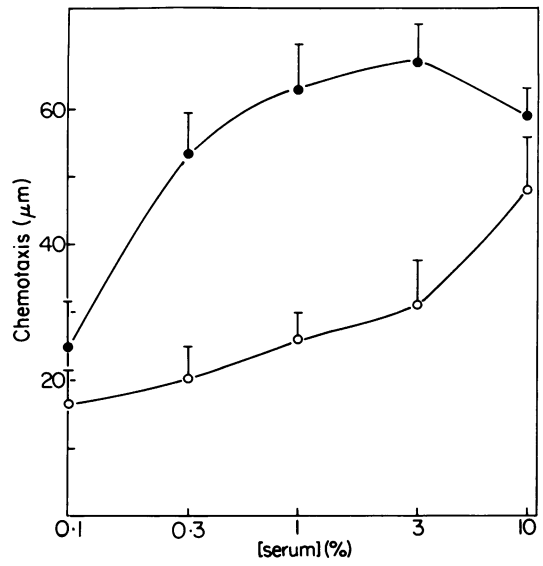

Figure 3. Chemotaxis as a function of serum concentration in the presence and absence of decomplemented synovial fluid. Data were taken from the legend to Table 1, except for the results with 0·1% serum, which were obtained during the experiments described in Table 1 but were not included in that table. (●) serum alone; (○) synovial fluid added.

Quantification and specificity

Two kinds of experiments were performed to quantify the inhibitor. In the first, measurements were made of chemotactic inhibition as a function of synovial fluid concentration (Fig. 2). Though these measurements showed only a modest degree of inhibition, this effect was found to correspond to a rather substantial quantity of inhibitor. This is shown in Fig. 3, which compares the distance moved in response to various dilutions of chemoattractant in the presence and absence of decomplemented synovial fluid. It can be seen in this figure, for example, that cells exposed to 3% zymosan-activated serum in the presence of synovial fluid migrated a substantially shorter distance than cells exposed to 0·3% serum alone. These results indicate that there was enough inhibitor in the fluid to neutralize well over 90% of the chemotactic activity present in 3% zymosan-activated serum. Nevertheless, the amount of inhibitor must be low relative to the quantity of complement-derived chemotactic factor potentially available in either serum or synovial fluids since little inhibition was seen when 10% serum was used as the attractant, and the inhibitor could not be detected at all unless the synovial fluid were decomplemented before use.

Other experiments suggested that the antagonist was specific for the chemotactic factor in zymosan-activated serum. Adding decomplemented synovial fluid to the neutrophil suspension during the chemotactic assay did not alter the response of the cells to zymosan-activated serum (Table 3), suggesting that the antagonist did not act against the cells *per se*, but somehow interfered with chemotaxis at the level of the chemotactic factor—i.e., that the antagonist was factor-directed, not cell-directed. Further evidence for an action against the chemotactic factor itself was the finding that the extent of inhibition of chemotaxis by synovial fluid depended on the identity of the chemotactic factor. In particular, decomplemented synovial fluid appeared to be at least as effective against purified $C5a_{des\ arg}$, the principal chemotactic factor in zymosan-activated serum (Zigmond & Hirsch, 1973), as it was against the zymosan-activated serum itself (Table 4), whereas it was without effect against the peptide chemoattractant f-met-leu-phe (Fig. 4).

Table 3. Chemotactic response of neutrophils after preincubation with decomplemented synovial fluid

Serum concentration (%)	Inhibition of chemotaxis (%)
10	−2·9±6·5
3	7·0±0·5
1	4·6±5·0
0·3	3·0±11·0

Neutrophil suspension (final concentration 10^6/ml) was incubated at 37° for 20 min with 10% hyaluronidase-treated (5 units/ml) decomplemented synovial fluid. This suspension was then placed in modified Boyden chambers and chemotaxis was measured as described in 'Materials and Methods', using zymosan-activated serum at the concentrations indicated as chemoattractant. Control assays were performed with neutrophils suspended in Gey's solution only and incubated at 37° for 20 min. Values for control chemotaxis (corrected for random migration of 52·5±1·4 μm) were 76·7±11·8, 79·7±7·3, 59·3±5·8, 22·0±5·5 μm, respectively, for 10%, 3%, 1% and 0·3% serum. Neutrophils mixed with synovial fluid gave the same random migration values as neutrophils mixed with Gey's solution only. The results are expressed as the mean ±1 SE of three experiments, each using a different synovial fluid specimen and neutrophils from a different donor.

Table 4. The effect of synovial fluid on the migration of neutrophils toward various chemoattractants

Chemoattractant	Inhibition of chemotaxis (%)
Activated serum, 1% (6)	58·9±5·5*
$C5a_{des\ arg}$, 1 μM (3)	82·7±2·6*

* Different from 0, $P < 0.001$.

Chemotaxis was measured in the presence and absence of 10% (v/v) decomplemented synovial fluid as described in Table 1, using zymosan-activated serum and $C5a_{des\ arg}$ as chemoattractants at the concentrations indicated. Values for control chemotaxis (corrected for random migration of 54·0±3·6 μm) were 72·5±6·8 μm for zymosan-activated serum and 43·3±11·3 for purified C5a. The number of experiments with each chemoattractant is given by the figure in parentheses. Each experiment was carried out using a different synovial fluid specimen and neutrophils from a different donor. Results are expressed as the mean±SE.

Further characterization

The chemotactic antagonist in synovial fluid appears to be a protein of mol. wt. ~25,000. That it is protein (or proteins) is indicated by the fact that it was destroyed when the decomplemented synovial fluid was boiled, or treated with trypsin, but was not affected when the fluid was treated with hyaluronidase (Table 5). In control experiments, it was shown that soybean trypsin inhibitor alone had no effect on the chemotactic inhibitor, and that hyaluronidase itself did not interfere with the chemotactic assay.

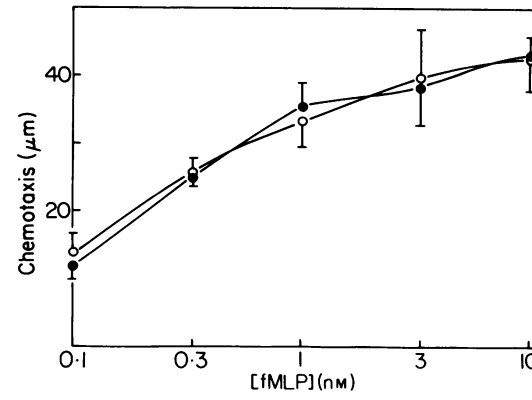

Figure 4. The effect of decomplemented synovial fluid on chemotaxis in response to f-met-leu-phe (fMLP). Chemotaxis was measured in the presence and absence of 10% (v/v) synovial fluid exactly as described in Table 1, except that f-met-leu-phe instead of zymosan-activated serum was used as chemotactic factor. The results are expressed as the mean ±1 SE of four experiments, each using a different synovial fluid specimen and neutrophils from a different donor. (●) f-met-leu-phe alone; (○) synovial fluid added.

The approximate size of the inhibitor was established by gel filtration of synovial fluid which had been pretreated with hyaluronidase to reduce its viscosity. On Sephadex G-150, the inhibitor was found to migrate roughly in the position of chymotrypsinogen A (Fig. 5). Because gel filtration separated the inhibitor from the much larger complement components, which had interfered with its detection in earlier experiments with native synovial fluid (Table 1), it was now possible to demonstrate it in the unheated fluid. As shown in the upper panel of Fig. 5, the native fluid contained a chemotactic inhibitor of the same potency and in the same location as the inhibitor found in the same fluid after decomplementation (Fig. 5, bottom). Thus, the chemotactic inhibitor is not generated as an artifact of decomplementation, but is an actual constituent of native synovial fluid.

DISCUSSION

The studies described in this paper demonstrate the

Table 5. Nature of the chemotactic inhibitor in synovial fluid

Treatment of synovial fluid	Inhibition of chemotaxis (%)
None	57·3±2·2
Boiling	4·0±2·3
Trypsin	8·3±8·8
Hyaluronidase	62·5±16·8

Decomplemented synovial fluid was used for these experiments. Boiled fluid was prepared by heating in boiling water for 3 min, then centrifuging at 800 *g* for 10 min to remove denatured proteins. Digestion with trypsin was carried out by mixing 1 ml of fluid with 0·1 ml freshly prepared trypsin in water (10 mg/ml), incubating for 10 min at 22°, then stopping proteolysis with 0·1 ml soybean trypsin inhibitor (15 mg/ml in water). Digestion with hyaluronidase was carried out by mixing 1 ml of fluid with 30 units of hyaluronidase and incubating for 30 min at 37°. A marked qualitative reduction in the viscosity of the synovial fluid indicated that hyaluronic acid had been degraded; no attempt was made, however, to measure the degradation of hyaluronic acid more precisely.

Chemotaxis was measured in the presence and absence of 10% (v/v) synovial fluid as described in Table 1, using 1% zymosan-activated serum as chemoattractant. The value for control chemotaxis (corrected for random migration of 50·0±3·3 μm) was 60·0±6·1 μm. Results are expressed as the mean ±1 SE of four experiments, each using different synovial fluid specimen and neutrophils from a different donor. Similar results were obtained at other serum concentrations.

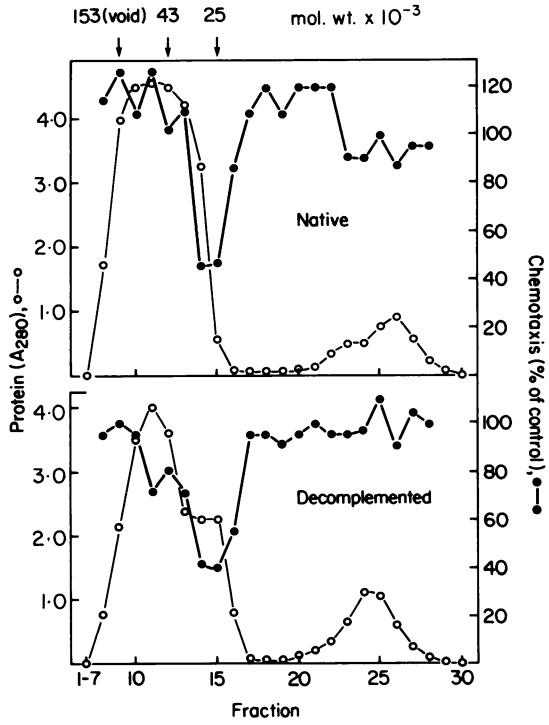

Figure 5. Chromatography of the chemotactic inhibitor of synovial fluid on Sephadex G-150. For details, see 'Materials and Methods'. The results shown are representative of four experiments, using two different synovial fluid specimens and neutrophils from four different donors. Above, native fluid. Control values were: chemotaxis 62 μm, random migration 68 μm. Below, decomplemented fluid. Control values were: chemotaxis 45 μm, random migration 57 μm. Controls were carried out as described in Table 1.

presence of chemotactic inhibitory activity in synovial fluid from patients with degenerative joint disease, a form of arthritis with little in the way of an inflammatory component. The activity is associated with one or more proteins of mol. wt. ~25,000. In unfractionated synovial fluid, activity could not be detected unless the fluid was first decomplemented by heat. The activity could, however, be demonstrated in unheated (native) synovial fluid after gel filtration to remove complement components, which were found to interfere with the assay. Recovery of inhibitory activity after gel filtration was similar for native and decomplemented synovial fluid, suggesting that the activity was stable to the decomplementing conditions (56° for 30 min).

The primary effect of this activity appeared to be against $C5a_{des\ arg}$-induced chemotaxis. The activity was not inhibiting cell locomotion *per se*, because it did not alter random migration. Its anti-chemotactic effect seemed to be directed specifically against C5a-related chemotaxis, since it had no effect on chemotaxis provoked by f-met-leu-phe, a member of another class of chemotactic factors (the N-formylated oligopeptides).

Hyaluronic acid, a major component of synovial fluid, has been reported to affect chemotaxis, increasing it modestly at low concentrations (Hakansson, Hallgren & Venge, 1980) and impairing it at high (Forrester & Wilkinson, 1981). In the present studies, however, hyaluronic acid was found to have little effect on neutrophil mobility. Random migration was the same in the presence of synovial fluid as in the presence of Gey's solution, and hyaluronidase treatment did not alter the effect of synovial fluid on chemotaxis toward zymosan-treated serum.

Chemotactic inhibitors from other sources have been described by several investigators. The inhibitory activity in joint fluid seems to be different from all of these. Its size distinguishes it from polymeric IgA, the material responsible for impaired chemotaxis in cirrhotics (Van Epps, Strickland & Williams, 1975), and from leukocyte inhibitory factor, a 70,000 mol. wt. antichemotactic protein released from stimulated lymphocytes (Rocklin, 1974; Rocklin, 1975). It is distinguished from the chemotactic-factor inactivators of serum (Ward & Ozols, 1969; Till & Ward, 1975), tissue extracts (Bronza & Ward, 1975) and the azurophil granules of neutrophils (Wright & Gallin, 1975; Wright & Gallin, 1977) by its stability at 56°, a temperature at which those inactivators are rapidly destroyed.

On the basis of the foregoing data, we propose that the chemotactic inhibitor in synovial fluid serves a regulatory role. It prevents the development of unprovoked inflammatory reactions in the joint space by antagonizing the chemotactic effects of small amounts of C5a that are likely to be released accidentally in the synovial fluid from time to time. Large quantities of C5a such as those released in response to a noxious agent would swamp the antichemotactic activity, however, so it would have little effect on such appropriately initiated inflammatory reactions.

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