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Complement C4-derived Monocyte-directed Chemotaxis-inhibitory Factor

A Molecular Mechanism to Cause Polymorphonuclear Leukocyte-predominant Infiltration in Rheumatoid Arthritis Synovial Cavities

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To reveal the mechanism of the lesser infiltration of monocytes in synovial cavities with rheumatoid arthritis despite the presence of chronic inflammation, the synovial fluid from 15 rheumatoid arthritis patients was analyzed with respect to leukocyte chemotaxis. The synovial fluid possessed strong chemotactic activity to polymorphonuclear leukocytes but rather suppressed one to monocytes. The synovial fluid contained two different inhibitory activities in monocyte chemotaxis. One, which also suppressed polymorphonuclear leukocyte chemotaxis, was identified as α_1 protease inhibitor. The other, with molecular weight of 8 kd, possessed the specificity to monocytes and shared the antigenicity with complement C4 but not with C3 or C5. A similar inhibitor was generated in normal human plasma when the classical pathway of the complement system was initiated with aggregated human IgG, while it was not when alternative pathway was initiated with zymosan. The small size factor in the synovial fluid, apparently derived from C4, seemed to be a cyto-directed factor that might block an early part of signal transduction system of monocytes in the chemotaxis. After removal of the small-size inhibitor, the synovial fluid exhibited chemotactic ability to monocytes. Therefore the apparent C4-derived factor might play a key role in the polymorphonuclear leukocyte-predominant infiltration in the synovial fluid of rheumatoid arthritis. (Am J Pathol 1991, 138:1279–1291)

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes progressive destruction of multiple joints. One of the causative factors of the joint destruction is a continuous infiltration of polymorphonuclear leukocytes (PMN) into the joint cavities.[1] Several authors reported that the leukocyte infiltration attributed to chemotactic factors such as complement-derived factors, C5a or C5a des Arg,[2,3] and a metabolite of arachidonic acid, LTB_4,[4,5] is generated in the synovial fluid of RA (RA-SF). However, because these chemotactic factors have been shown to be attractive to monocytes as well, at least *in vitro*,[6–9] it is difficult to explain satisfactorily the characteristic feature of RA-SF with the PMN-predominant infiltration, despite the presence of chronic inflammation. In this regard, we speculated on the existence of some inhibitory activity, specific to monocyte chemotaxis in RA-SF, that might contribute to the PMN-predominant infiltration. In the present study, we initially confirmed the property of leukocyte infiltration in 15 RA patients then demonstrated a significant inhibitory capacity to blood monocyte chemotaxis in the RA-SF. Finally we separated two different inhibitory molecules from the RA-SF and characterized them by means of immunology and biochemistry.

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Materials and Methods

Synovial Fluid

Synovial fluid was collected from knee joints of 15 RA patients and 10 osteoarthritis (OA) patients for the sake of contrast, using the sterilizing arthrocentesis technique.[10] Count of total leukocyte number and analysis of the cells in the synovial fluid were immediately made. All of the synovial fluid remaining was immediately centrifuged at 3000 rpm for 20 minutes and the supernatant was stored at −70°C.

Reagents

Heparin, zymosan A, bovine serum albumin (BSA), α_1 protease inhibitor (α_1-PI), cytochrome C (Cyt-C), Phosphorylase B (Phos-B), N-formyl-Met-Leu-Phe (fMLP), Cohn II, III fraction from human plasma, and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Company (St. Louis, MO). Lymphocyte-separating medium (LSM) and antisera against complement components C3, C4, and C5 or against α_1-PI were purchased from Organon Teknica Company (Cappel Products, West Chester, PA). Another antiserum against complement C4 was purchased from Behring-Werke Company (Marburg, Germany). Antiserum against human C3 proactivator (factor B) was obtained from Binding Site Ltd. (Birmingham, UK). Hank's balanced salt solution (HBSS) and Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Nissui Company (Tokyo, Japan). Hyaluronidase L and Dextran T 500 were purchased from Nakarai Company (Kyoto, Japan). Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). CM-cellulose CM52 was purchased from Whatman Company (Springfield Mill, UK). Toyopearl HW-50S and HW-55S were obtained from Tosoh Company (Tokyo, Japan) and the former gel was packed in a high-pressure glass column (GCV-20; Umetani, Tokyo, Japan) using a high-performance liquid chromatography (HPLC) system (LKB, Pharmacia). A multiwell chamber for chemotaxis assay was obtained from Neuro Probe Company (Bethesda, MD). Mercaptomethyl-guanidoethylthiopropanoic acid (mercapto-guanido compound), which is a specific inhibitor of carboxypeptidase N, was purchased from Calbiochem, Behringer-Diagnostics (La Jolla, CA). Ethylenediamine tetraacetic acid disodium salt (EDTA) was purchased from Dojindo Laboratories (Kumamoto, Japan). Nucleopore filters for the multiwell chamber assay were purchased from Nuclepore Company (Pleasanton, CA). Ninety-six–well Micro Test III tissue culture plates (Falcon 3070) were obtained from Becton Dickinson Labware (Oxnard, CA). Spectra/Por 6 dialysis tube was purchased from Medical Industries Inc. (Los Angels, CA).

Activation of Classical or Alternative Pathway of Complement System

To activate the alternative pathway of the complement system, normal human plasma containing 4 mmol/l (millimolar) EDTA and 10 μmol/l (micromolar) mercapto-guanido compound was treated with zymosan A (20 g/l) by an incubation at 37°C for 60 minutes, as described by Hugli et al,[11,12] with some minor modifications. The zymosan-activated plasma (ZAP), which contained the chemotactic factor C5a, was ordinarily used as the chemoattractant in the chemotaxis-inhibitory assays as well. Heat-aggregated IgG was prepared by exposing the Cohn II, III fraction of normal human plasma in 0.02 mol/l (molar) phosphate-buffered saline (PBS) at pH 7.2 to 64°C for 20 minutes, as described by Hugli et al.[12] Normal human plasma containing 4 mmol/l EDTA and 10 μmol/l mercapto-guanido compound was treated with the heat-aggregated IgG (0.5g/l) by an incubation at 37°C for 60 minutes to activate the classical complement pathway, as described by Hugli et al,[12] with some minor modifications, and used as aggregated IgG- activated plasma.

Purification of Antibody

IgG fraction of antisera against human complement components C3, C4, C5, and factor B or against α_1-PI was prepared, using the fractionation with 40% ammonium sulfate followed by Toyopearl HW-55S gel filtration column chromatography. The quality of each of the antibodies was examined using the double-immunodiffusion method of Ouchterlony.[13] Because each of them made a single precipitation line against normal human plasma, they were apparently monospecific.

Preparation of PMNs and Mononuclear Leukocytes (MNLs)

Polymorphonuclear leukocytes and MNLs were prepared from heparinized healthy human venous blood by the gradient centrifugation method using LSM as described by Böyum.[14] Briefly, heparinized venous blood was mixed with 6% Dextran T 500 (five parts blood, one part dextran solution) and maintained at room temperature for 40 minutes. The leukocyte-rich supernatant of this mixture was layered on the same volume of LSM and

centrifuged at 300*g* for 30 minutes at room temperature. Polymorphonuclear leukocytes sedimented at the bottom of the tube and MNLs enriched at the interface between plasma and LSM were collected in HBSS (pH 7.0) separately, washed twice with HBSS containing 4 mmol/l EDTA and 0.5% BSA, then once with HBSS containing 0.5% BSA to remove platelets. Polymorphonuclear leukocytes and MNLs were resuspended in HBSS containing 0.5% BSA at the concentration of 6×10^6 cells/ml or in RPMI 1640 containing 10% heat-inactivated fetal calf serum at the concentration of 1×10^6 cells/ml for the morphologic polarization assay or for multiwell chamber assay, respectively.

Morphologic Polarization Assay in Chemotaxis

Morphologic polarization assay was performed according to the method of Cianciolo and Snyderman,[15] with minor modifications. Briefly, the cell suspension was incubated with samples for 10 minutes at 37°C in a polypropylene tube. Immediately after the incubation, 1 ml of cold parafolmaldehyde (8% w/v) buffered with 0.1 mol/l phosphate (pH 7.2) was added to each tube to fix the cells. After fixing for 1 hour at 4°C, the suspensions of PMNs were stained with Türk staining. The MNLs were centrifuged further mildly for 10 minutes and washed twice with PBS. The fixed cells then were stained cytochemically for the nonspecific esterase with α-naphtyl acetate as the substrate for 50 minutes at 37°C to identify monocytes in the MNLs and washed twice with PBS. The cell suspensions thus treated were deposited into the wells of 96-well tissue culture plates. The number of polarized and nonpolarized cells of PMNs or monocytes was counted at least until 200 using an inverted microscopy (IMT-2, Olympus Optical Co., Tokyo, Japan) with or without the refractive interference method. The activity of the samples was expressed as the percentage of the monocyte number with polarized morphology against the total number of monocytes counted. Inhibitory activity was displayed as percentage of inhibition compared to the monocyte-polarized ratio when maximally stimulated by ZAP, which was calculated by the following formula

$$\text{Inhibition} = \left(1 - \frac{\text{polarized ratio with ZAP plus inhibitor}}{\text{polarized ratio with ZAP}}\right) \times 100.$$

Multiwell Chamber Assay in Chemotaxis

Multiwell chamber assay was performed as described by Falk et al[16] using the Nucleopore filter with pore sizes of 3 μm and 5 μm for PMNs and MNLs, respectively, in the 48-well multiwell chamber; the volume of the lower compartment for chemoattractant was 25 μl and of the upper compartment for indicator cells was 50 μl. The chemotactic activity was expressed as the mean number of migrated cells at five high-power fields of a microscope in duplicate samples.

Immunoadsorption of Chemotaxis-inhibitory Activity

IgG antibodies against complement C3, C4, C5, and factor B, or against α_1-PI were coupled to cyanogen bromide-activated Sepharose 4B beads by the method of Porath et al.[17] The adsorption experiment was performed by a batch-wise method. Samples were incubated with these immunoadsorbent gel beads in PBS for 30 minutes at room temperature. After centrifugation at 10,000 rpm for 5 minutes, the supernatants were separated and used as the samples in chemotaxis assay.

Estimation of Molecular Weight of the Inhibitors to Monocyte Chemotaxis in RA-SF

To estimate the molecular weight of the inhibitors to monocyte chemotaxis, 3 ml of RA-SF was subjected to the gel filtration column chromatography with Toyopearl HW-55S gel (column size, Φ 2.5cm × 65 cm; bed volume, 319 ml) that had been equilibrated with PBS. To decrease the viscosity, the synovial fluid was pretreated with hyaluronidase (10 U/ml) for 20 minutes at 37°C before the gel filtration. Fractions of the column chromatography with Toyopearl HW-55S were assayed for the inhibitory activity to monocyte chemotaxis with ZAP, using the polarization method as described above.

Purification of Small Molecular Size Chemotaxis-inhibitory Factor in RA-SF

Of RA-SF, 300 ml were fractionated with ammonium sulfate and the supernatant fraction of 80% ammonium sulfate saturation was dialyzed against 0.1 mol/l ammonium formate (pH 5.0), concentrated using a centrifuging evaporator (Savant, Hicksville, NY) and applied to a CM-cellulose (CM52) ionic exchange column (size, Φ 1 cm × 26 cm; bed volume, 20.4 ml) equilibrated with 0.1 mol/l ammonium formate (pH 5.0) containing 0.5 mmol/l EDTA. Inhibitory fractions eluted in the washing process with the initial buffer following to the breakthrough fraction were pooled and concentrated using

the centrifuging evaporator to 2 ml. Of the concentrated sample, 2 ml were applied to the HPLC system with the Toyopearl HW-50S gel filtration column (size, Φ 2 cm × 100 cm; bed volume, 314 ml) equilibrated with PBS.

Anaphylatoxin Assay

Anaphylatoxin activity was measured by the smooth muscle contraction assay with estrous uterus of Sprague-Dawley rats, as described by Cochrane et al,[18] with some modifications.

Statistical Analysis

A paired or nonpaired t test was performed.

Results

Profile of Patients and the Characteristics of Inflammation in RA and Osteoarthritis (OA)

Fifteen patients (1 man, 14 women) ranging in age from 38 to 77 years (average, 54.2 years) with RA diagnosed according to the revised criteria of the American Rheumatism Association were examined.[19] Table 1 demonstrates the clinical characteristics of RA and OA patients. All patients with RA and of OA were being treated with some nonsteroidal anti-inflammatory agents. Eight, three, two, and four individual with RA also were treated with intramuscular injections of a gold-containing compound, oral administrations of D-penicillamin, oral administrations of bucillamine, and oral administrations of predonisolone, respectively. Patients who had been treated with intra-articular injection of corticosteroid within the recent 1-month period were excluded. The average value of erythrocyte sedimentation rate at 1 hour and serum C-reactive protein of the RA patients were 68.4 ± 23.2 mm and 2.4 ± 1.6 mg/dl, respectively. The average number of leukocytes infiltrated in RA-SF and in the synovial fluid of OA patients (OA-SF) were 12,700 and 860, respectively. The proportions of PMNs in RA-SF and OA-SF were 78.8% and 11.2%, respectively. Thus one of the characteristic features of RA observed in the present study was severe PMN-predominant infiltration in the synovial cavity. This observation was consistent with previous reports.[20]

Table 1. *Clinical and Laboratory Characteristics of Patients with Rheumatoid Arthritis (RA) and Osteoarthritis (OA)*

Characteristics	RA	OA
No. of cases	15	10
Sex (male/female)	1/14	4/6
Age(yrs) (average)	38–77 (54.2)	59–89 (76.3)
Duration of disease(yrs) (average)	3–18 (10.3)	
Stage(I/II/III/IV)	3/5/7/0	
C reactive protein (mg/dl) (mean ± standard deviation(SD))	2.4 ± 1.6	<0.3
Erythrocyte sedimentation rate at 1 hour (mm) (mean ± SD)	68.4 ± 23.2	<20
Therapy		
Nonsteroidal anti-inflammatory agents	15	10
Gold compound	8	0
D-penicillamine	3	0
Bucillamine	2	0
Corticosteroid	4	0
Synovial fluid		
No. of leukocytes (average)	5250–43,700 (12,700)	0–1250 (330)
PMN(%) (average)	65–95 (78.8)	0–20 (11.2)
Monocytes(%) (average)	3–25 (12.1)	13–54 (32.7)

PMN, polymorphonuclear leukocytes.

Chemotactic Potency of RA-SF and OA-SF to Monocytes and PMNs

Before investigating the inhibitory capacity of RA-SF for monocyte chemotaxis, we examined the chemotactic potency of RA-SF using the morphologic polarization assay (Table 2). Tenfold diluted RA-SF caused the polarized shape change of the cells by 87.9% ± 13.5% (mean ± standard deviation [SD]) and 18.4% ± 5.6% of PMNs and monocytes, respectively. In contrast, tenfold diluted OA-SF caused a change by 18.4% ± 5.6% and 19.8% ±

Table 2. *Chemotactic Activities to PMNs and to Monocytes in the Synovial Fluid (SF) of Patients with Rheumatoid Arthritis (RA) or with Osteoarthritis (OA)*

	Cells polarized(%)	
Samples	PMNs (mean ± SD)	Monocytes (mean ± SD)
ZAP	88.2 ± 9.6	43.7 ± 5.9
PBS	8.3 ± 2.5	7.4 ± 4.2
RA-SF (n = 15)	87.9 ± 13.5 *	17.2 ± 12.4
OA-SF (n = 10)	19.4 ± 12.0 *	23.6 ± 15.6

PMNs, polymorphonuclear leukocytes; ZAP, 30 μl of 10-fold diluted zymosan activated plasma in Hank's balanced salt solution (HBSS) (100-fold diluted at the final) as positive control; PBS, 30 μl of phosphate-buffered saline as negative control (PBS and HBSS caused polarization in similar extend); RA-SF, 30 μl of crude synovial fluid of RA patients (10-fold diluted at the final); OA-SF, 30 μl of crude synovial fluid of OA patients (10-fold diluted at the final); SD, standard deviation (n = 5); cells polarized(%), polarized cells that are reported as a percentage of the total(%). Chemotactic activities were measured by the morphologic polarization method (total volume of the assay, 300 μl) as described in Materials and Methods.
* Statistical difference with $P < 0.01$ (nonpaired *t*-test).

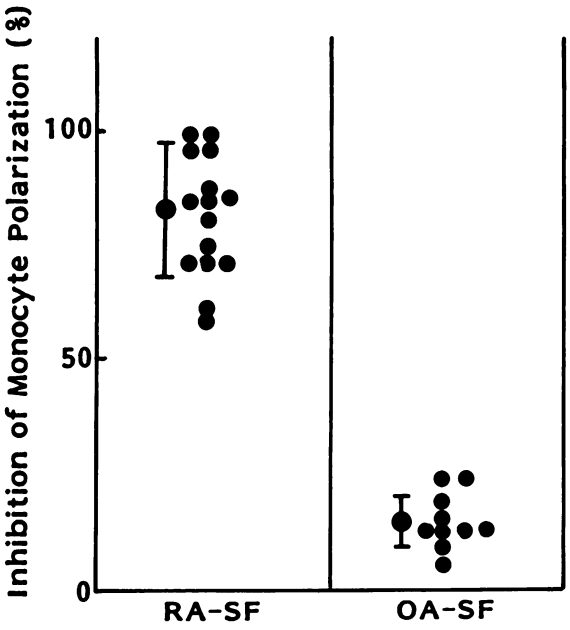


Figure 1. *Inhibitory capacity to monocyte chemotaxis caused with 100-fold diluted zymosan-activated plasma (ZAP) in the synovial fluid of rheumatoid arthritis (RA-SF). RA-SF possesses much stronger inhibitory capacity to monocyte chemotaxis (81.4% ± 13.5%) than the synovial fluid of osteoarthritis (OA-SF) (13.7% ± 5.2%). There was a statistical difference in the inhibitory capacity to monocyte chemotaxis between RA-SF and OA-SF (P < 0.01; nonpaired Student's t-test).*

7.0% of PMNs and monocytes, respectively. There was a statistical difference for the chemotactic potency to PMNs between that of RA-SF and OA-SF, but not to monocytes. Because the polarized shape change is the initial step of the chemotactic reaction, RA-SF possessed the chemotactic activity selective to PMNs rather than to monocytes. This result correlates with one of the characteristic features of RA, ie, PMN-predominant cell infiltration into the joint cavities.

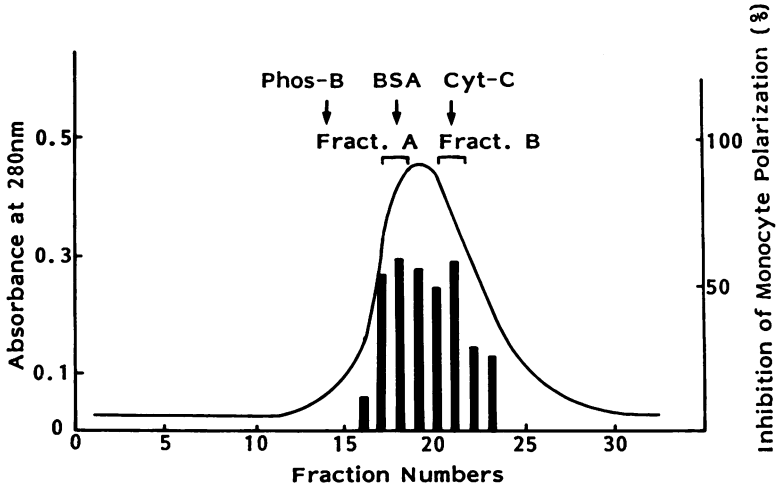
Inhibitory Capacity to Monocyte Chemotaxis in RA-SF

Figure 1 demonstrates that a coexistence of 3% (v/v) of RA-SF in the suspension of MNLs suppressed 81.4% ± 13.5% (mean ± SD) of the monocyte shape change attracted by the maximal stimulation with ZAP in the morphologic polarization assay. In contrast, OA-SF only suppressed it by 13.7% ± 5.2%. There is a statistical difference in the inhibitory capacities of RA-SF and OA-SF ($P < 0.01$). These data indicate the presence of inhibitory activity to monocyte chemotaxis in RA-SF. However there is no clear correlation between the intensity of inhibitory activity *in vitro* and either the actual number or the proportion of macrophages infiltrated in each RA-SF. Although the RA-SF itself possessed the weak chemotactic potency to monocyte *in vitro*, as described above, it did not significantly affect the chemotactic inhibition assay at the concentration of RA-SF as low as 3% (v/v).

Separation of Two Different Inhibitory Molecules to Monocyte Chemotaxis in RA-SF

To reveal the nature of the inhibitory activity to monocyte chemotaxis in RA-SF, an aliquot of RA-SF pretreated with hyaluronidase was subjected to the gel filtration column chromatography with Toyopearl HW-55S. Figure 2 is a typical chromatographic pattern in five similar experiments. The inhibitory activities to the monocyte chemotaxis were eluted with two peaks. To exclude the possible cross-contamination by each other, each of the fractions, fraction A and B in Figure 2, was further chromatographed using the HPLC system with the gel permeation

Figure 2. *Elution profile of the inhibitory activities to monocyte chemotaxis in RA-SF on the molecular sieve column chromatography with Toyopearl HW-55 gel. Of RA-SF, 3 ml was subjected to the molecular sieve column (ϕ 2.5 cm × 65 cm; bed volume, 319 ml). There were two inhibitory peaks with the apparent molecular sizes 64 kd and less than 12 kd, respectively. The solid line denotes absorbance at 280 nm. The closed columns denote inhibitory activity to monocyte chemotaxis caused with 100-fold diluted ZAP in the morphologic polarization assay. Phos-B, phosphorylase B; BSA, bovine serum albumin; Cyt-C, cytochrome C.*



Toyopearl HW-50S column (size, Φ 2 cm × 100 cm; bed volume, 314 ml). Figure 3 illustrated the HPLC elution profile of fraction B. In this step, fraction B was separated clearly from that in fraction A. The inhibitory activity of fraction B was eluted at the position between that of cytochrome C and l-tyrosine as molecular weight markers, and the molecular size was estimated to be about 8 kd from a standard curve of molecular weight with markers. This fraction (fraction B' in Figure 3) suppressed only monocytes, while the suppressive effect was not affected by the chemoattractant used, such as ZAP and fMLP. On the other hand, fraction A obtained with HPLC suppressed the chemotaxis of not only monocytes but also PMNs caused by either ZAP or fMLP (data not shown).

Identification of the High–Molecular-weight Inhibitor: Effect of Anti–α_1-PI Antibody

α_1-PI and Bb fragment (breakdown product of complement factor B) were reported to be potent inhibitors of leukocyte chemotaxis.[21–23] Therefore we examined the effect of anti–α_1-PI and anti-factor B antibodies to the inhibitory activities to leukocyte chemotaxis in RA-SF. As shown in Table 3, anti–α_1-PI antibody but not anti-factor B antibody markedly diminished the inhibitory activity of fraction A to leukocyte chemotaxis caused by ZAP as well as by fMLP. However neither anti–α_1-PI nor anti-factor B antibody affected that of fraction B' on the monocyte chemotaxis caused by either of the chemoattractants. This result indicated that the inhibitory activity in fraction A shared the same antigenicity with α_1-PI.

Effect of α_1-PI to Leukocyte Chemotaxis

Plasma α_1-PI purchased commercially was purified further by the gel filtration column chromatography with Toyopearl HW-55 gel. The purified material showed a single protein band in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (data not shown). Figures 4 and 5 demonstrate the inhibitory effects of the purified α_1-PI to chemotaxis of PMNs and monocytes. It suppressed the PMN chemotaxis caused with ZAP in a dose-dependent manner at the concentrations of between 1 μg/ml and 30 μg/ml (Figure 4). It also suppressed the monocyte one but in a different dose-dependent manner at concentrations higher than 3 μg/ml (Figure 5). Furthermore it suppressed the chemotaxis of both PMNs and monocytes caused with fMLP in similar dose-dependent manners (data not shown). These results indicated that α_1-PI itself possessed the inhibitory activity to leukocyte chemotaxis in the morphologic polarization assay. From these results, it was concluded that the inhibitory activity in fraction A was α_1-PI itself or its slightly modified derivative.

Purification of Low–Molecular-weight Chemotaxis-inhibitory Factor in RA-SF

The low–molecular-weight inhibitor to monocyte chemotaxis in RA-SF was purified by ammonium sulfate fractionation, CM-cellulose column chromatography, and gel permeation HPLC, in this order. The low–molecular-weight inhibitor was fractionated into the supernatant of 80% ammonium sulfate saturation and was

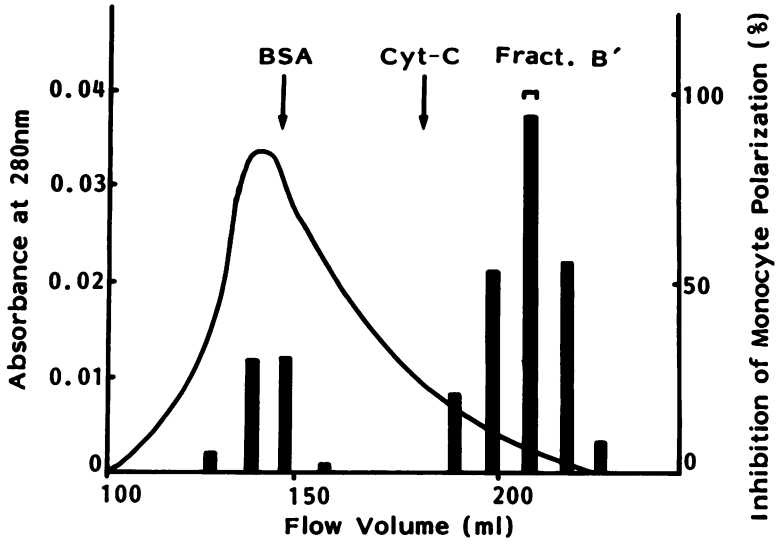


Figure 3. *Molecular sieve column chromatography using HPLC system with Toyopearl HW-50S gel of the fraction B in Figure 2. The pooled sample of fraction B in Figure 2 was concentrated to 1.5 ml and applied to the column (size, ϕ 2 cm × 100 cm; bed volume, 314 ml). The main peak of the inhibitory activity to monocyte chemotaxis was eluted at a position between the cytochrome C and tyrosine as molecular-weight markers. The eluted position of tyrosine is not shown because it is out of the graph. The apparent molecular weight of the inhibitor was estimated to be 8 kd. The solid line denotes absorbance at 280 nm. The closed columns denote inhibitory activity to monocyte chemotaxis caused with 100-fold diluted ZAP in the morphologic polarization assay. Cyt-C, cytochrome C.*

Table 3. *Effect of Antibody Against Alpha-1-protease Inhibitor on the Inhibitory Activities to Leukocyte Chemotaxis in RA-SF*

Stimulant		Inhibitor	Antibody gel	Cells polarized(%) PMNs	Monocytes
ZAP				86.8 ± 4.1	44.3 ± 3.6
PBS				6.5 ± 4.3	7.4 ± 2.2
ZAP	+	Fraction A(40% v/v)	Rabbit IgG	32.8 ± 5.9	16.7 ± 4.7
ZAP	+	Fraction A(40% v/v)	Anti-factor B	34.6 ± 3.2 *	16.3 ± 4.3 *
ZAP	+	Fraction A(40% v/v)	Anti-α_1-PI	86.4 ± 2.3	44.2 ± 3.9
ZAP	+	Fraction B'(40% v/v)	Rabbit IgG	87.2 ± 4.3	12.3 ± 2.1
ZAP	+	Fraction B'(40% v/v)	Anti-factor B	83.7 ± 4.1	11.5 ± 2.3
ZAP	+	Fraction B'(40% v/v)	Anti-α_1-PI	84.5 ± 2.1	11.8 ± 1.6

Fraction A and fraction B', pooled fractions of the molecular sieve column chromatography with HW-55 gels and HW-50S gels, as demonstrated in Figures 2 and 3, respectively. Anti-α_1-PI, anti-factor B, or Rabbit IgG, IgG fraction of anti-human α_1-PI rabbit antiserum, anti-human factor B goat antiserum or normal rabbit serum immobilized on Sepharose 4B beads, respectively.

* Statistical difference with $P < 0.01$ (paired *t*-test). An aliquot, 120 μl, of the fraction A or the fraction B' was pretreated with the antibody affinity gels at room temperature for 60 minutes in a batch-wise method. After centrifugation, 120 μl of the supernatant were subjected to the polarization inhibition assay. Values are mean ± SD (n = 5).

separated from α_1-PI and the chemotactic activity to PMNs in RA-SF in this step. The supernatant fraction also contained the anaphylatoxin activity to rat estrous uterus. Figure 6 demonstrates the elution profile in the CM-cellulose column chromatography. The major inhibitory activity was eluted in the washing fraction with the initial buffer following the breakthrough fraction and was separated from the bulk of proteins in this step. A part of the anaphylatoxin activity was coeluted with the inhibitory activity again. In the gel permeation column chromatography with HPLC, the inhibitory activity was coeluted with a single major protein peak at the position between that of cytochrome C and l-tyrosine as the molecular weight markers (Figure 7). The molecular weight of the inhibitor, therefore, was estimated to be about 8 kd. The anaphylatoxin activity disappeared in the gel filtration step. The molecule purified was not so sensitive to a protein staining as visualized in the polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and, therefore, the purity of the final preparation was not confirmed.

Appearance of Chemotactic Activity of RA-SF to Monocyte After Removal of Low–Molecular-weight Inhibitor by Ammonium Sulfate Fractionation

As described above, the low–molecular-weight inhibitor in RA-SF was separated from the major chemotactic activity to PMNs by the ammonium sulfate fractionation. To elucidate the contribution of the low–molecular-weight inhibitor to the lesser monocyte infiltration in RA-SF, we examined the chemotactic activity to monocytes in a 40% to 80% fraction using the morphologic polarization assay. After dialysis against PBS with a dialysis tube (molecular sieve 2000), the volume of the 40% to 80% fraction as well as the supernatant fraction of 80% ammonium sulfate saturation was adjusted to that of the initial RA-SF, and the chemotactic activity to monocytes was measured by the morphologic polarization assay. As shown in Table 4, the 40% to 80% fraction now possessed a strong chemotactic activity to monocytes. The apparent capacity of the fraction for the monocyte chemotaxis was much stronger than the initial RA-SF with a statistical difference ($P < 0.01$). The supernatant fraction exhibited no chemotactic activity. These data indicated that the lesser chemotactic activity of RA-SF to monocytes was, at least partly, due to the presence of the low–molecular-weight inhibitor.

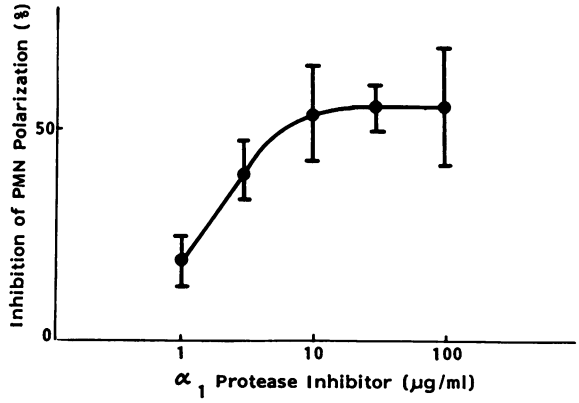


Figure 4. *Effect of α_1 protease inhibitor (α_1-PI) on polymorphonuclear leukocyte (PMN) chemotaxis. PMNs, 6 × 10^5 cells/ml in Hank's balanced salt solution (pH 7.2) containing 0.5% bovine serum albumin, were attracted to polarize with 100-fold diluted ZAP in the presence of various concentrations of α_1-PI. The inhibitory activity of α_1-PI was obtained as a percentage of inhibition using the formula as described in Materials and Methods. Values are mean ± standard deviation(SD) in five experiments.*

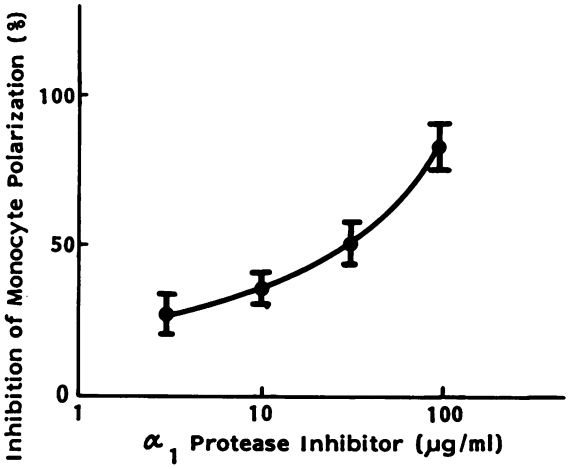


Figure 5. *Effect of α_1-PI on monocyte chemotaxis. Monocytes, 6×10^6 cells/ml in HBSS containing 0.5% BSA, were attracted to polarize with 100-fold diluted ZAP in the presence of various concentrations of α_1-PI. The inhibitory activity of α_1-PI was obtained as a percentage of inhibition using the formula as described in Materials and Methods. Values are mean ± SD in five experiments.*

Functional Characteristics of the Low–Molecular-weight Inhibitor in Monocyte Chemotaxis

Table 5 demonstrates the effect of the low–molecular-weight factor purified to monocyte chemotaxis caused with the various chemoattractants. The inhibitor suppressed the monocyte chemotaxis when maximally stimulated with either ZAP or fMLP in both the multiwell chamber assay and the morphologic polarization assay. In comparison to this, it did not suppress the chemotaxis caused with PMA, which is a well-known activator of protein kinase C in a signal transduction system of mammalian cells. The purified inhibitor did not suppress the chemotaxis of PMNs again when stimulated with either of the chemoattractants ZAP or fMLP (data not shown). The inhibitor itself did not attract the chemotaxis of monocytes (Table 4) or PMNs (data not shown).

Cell-directed Property of the Low–Molecular-weight Inhibitor in Monocyte Chemotaxis

The inhibitors of chemotaxis generally are classified into two categories such as a cell-directed inhibitor and chemoattractant-directed one. Because the low–molecular-weight inhibitor suppressed the monocyte chemotaxis caused with either of ZAP or fMLP, the inhibitor was assumed to be a cell-directed factor. We confirmed this by a preincubation experiment as follows, and the results are shown in Table 6. When monocytes were preincubated with the inhibitor at 37°C for 30 minutes, washed twice with PBS, and then attracted the locomotion with either ZAP or fMLP, the cells could not display the normal chemotactic response in the morphologic polarization assay. The pretreatment of monocytes with the low–molecular-weight inhibitor did not affect the viability of the cells at all when examined using the trypan blue dye exclusion assay (data not shown). In the multiwell chamber assay, the effect by the pretreatment was not so clear as in the polarization assay (data not shown). This was probably due to the requirement of a long incubation period in the former assay in which the cell could regenerate the machinery needed for the chemotaxis that had been blocked once by the inhibitor.

Immunoadsorption of the Low–Molecular-weight Inhibitor by Anti-complement Antibodies

Because the low–molecular-weight inhibitor was cofractionated with an anaphylatoxin activity in the ammonium

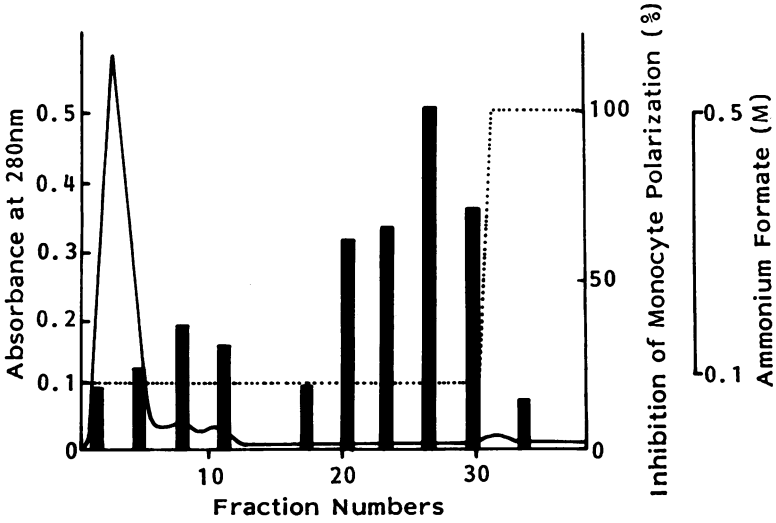


Figure 6. *Ionic exchange column chromatography with CM-cellulose of the low–molecular-weight inhibitor to monocyte chemotaxis. The column was equilibrated with 0.1 mol/l ammonium formate buffer (pH 5.0) containing 0.5 mmol/l ethylenediamine tetraacetic acid. After washing with the equilibration buffer, a stepwise elution with 0.5 mol/l ammonium formate buffer (pH 5.0) was performed. Inhibitory activity to monocyte chemotaxis was pooled as indicated. The solid line denotes absorbance at 280 nm and the closed columns denote the inhibitory activity to monocyte chemotaxis caused with 100-fold diluted ZAP. The dotted line denotes measured concentration of ammonium formate.*

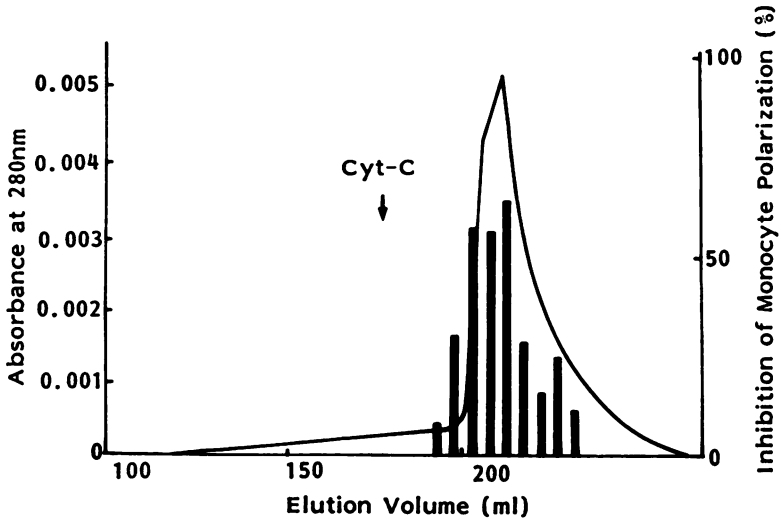


Figure 7. *Separation of the low–molecular-weight inhibitor in the pooled fraction of CM-cellulose column chromatography by high-performance liquid chromatography with Toyopearl HW-50S gel. The elution buffer was phosphate-buffered saline. The pooled fraction as shown in Figure 5 was concentrated to 2 ml, dialyzed against PBS, and applied to the column (size, ϕ 2 cm × 100 cm; bed volume, 314 ml). The solid line denotes absorbance at 280 nm and the closed columns denote the inhibitory activity to the monocyte chemotaxis caused with 100-fold diluted ZAP in the morphologic polarization assay.*

sulfate fractionation as well as CM-cellulose column chromatography, we investigated the effect on the inhibitor of the monospecific antibodies against the precursor molecules of anaphylatoxins in the complement systems such as C3, C4, and C5. Table 7 demonstrates the result of a batch-wise experiment using the IgG antibodies immobilized on Sepharose 4B beads. The anti-C4 antibody Sepharose 4B beads, but not other beads, absorbed the inhibitory activity of the low–molecular-weight inhibitor. To preclude the possibility that the anti-C4 antibody used was not really monospecific, we used another antibody from a different source and confirmed the absorption by anti-C4 antibody beads, as shown as experiment 2 in Table 7. These results indicate that the low–molecular-weight inhibitor shared the antigenicity with complement C4, and, therefore, the inhibitor seemed to be a breakdown product of complement C4.

Generation of Similar Chemotaxis Inhibitory Activity in Plasma by Activation of Classical Complement Pathway

In the light of the molecular weight and the antigenicity of the low–molecular-weight inhibitor in RA-SF, we speculated that the C4-derived inhibitory factor might be generated in the activation of classical complement pathway occurring in RA-SF. Therefore we investigated the generation of the inhibitory factor in the plasmas of which either the classical or the alternative complement pathway was activated. The supernatant fraction of 80% ammonium sulfate saturation of the aggregated IgG-activated plasma (the classical pathway activation) suppressed the monocyte chemotaxis caused with ZAP. However it did not suppress the PMN chemotaxis (data not shown). On the other hand, the same fraction of ZAP (the alternative pathway activation) had no inhibitory activity to monocyte chemotaxis (Table 8). Furthermore the inhibitory activity in the fraction of aggregated IgG-activated plasma was absorbed by the anti-C4 antibody on Sepharose 4B beads but not by the anti-C3 or anti-C5 antibody beads (Table 9). These results indicated that the activation of the classical complement pathway generated an inhibitor similar to that observed in RA-SF, and that the alternative pathway could not substitute for each other.

Discussion

In the present study, we observed the strong inhibitory activity to monocyte chemotaxis in the RA-SFs of all of the

Table 4. *Appearance of Chemotactic Activity to Monocytes After Removal of Low–Molecular-weight Inhibitor by Ammonium Sulfate Fractionation*

Stimulant	Cells polarized(%) (mean ± SD) (n = 4)	
PBS	6.4 ± 2.1	
ZAP	42.5 ± 4.7	
crude RA-SF	19.0 ± 3.3	
80% sup	6.0 ± 2.1	*
40%–80% ppt	33.7 ± 3.3	

PBS, phosphate-buffered saline; ZAP, zymosan-activated plasma.

RA-SF, 30 μl of the synovial fluid of rheumatoid arthritis (10-fold diluted at the final); 80% sup, supernatant fraction with 80% saturated ammonium sulfate of synovial fluid with rheumatoid arthritis (10-fold diluted at the final); 40%–80% ppt, precipitated fraction of 40%–80% ammonium sulfate saturation fractionation of synovial fluid with rheumatoid arthritis.

* Statistical difference with $P < 0.01$ (paired t-test).

After the precipitated fraction was dissolved into water, the fractions of the ammonium sulfate cut were dialyzed against PBS with a Spectra/Por 6 dialysis tube (molecular sieve, 2000) and adjusted the volume to that of initial RA-SF, before the morphological polarization assay. After that, 30 μl of each fraction was used in the morphologic polarization assay (10-fold diluted at the final).

Table 5. *Effect of Low-Molecular-weight Chemotaxis Inhibitory Factor in RA-SF to Monocyte Chemotaxis Caused with Various Chemoattractants*

Stimulant	Chamber assay (Migrated cell numbers at 5HPFs) (mean ± SD) (n = 5)	Polarization assay (Cells polarized(%)) (mean ± SD) (n = 5)
ZAP†	665 ± 24 *	49.2 ± 1.5 *
ZAP + Inhibitor	173 ± 9 *	16.6 ± 2.3 *
fMLP†	736 ± 38 *	48.9 ± 0.9 *
fMLP + Inhibitor	213 ± 12 *	21.8 ± 2.0 *
PMA†	725 ± 13	42.7 ± 0.3
PMA + Inhibitor	714 ± 26	42.1 ± 1.4
PBS†	8 ± 3	7.5 ± 2.6

ZAP, zymosan-activated plasma; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline.
fMLP, 30 μl of 10^{-7} mol/l formyl-Met-Leu-Phe in HBSS (at the final concentration 10^{-8} mol/l); PMA, 30 μl of 10^{-8} mol/l phorbol myristate acetate in HBSS (at the final concentration 10^{-9} mol/l); inhibitor, 120 μl of the pooled fraction in the gel permeation HPLC as shown in Figure 6 (at the final concentration A_{280} = 0.006); 5HPFs, five fields selected at random in microscope observation with high-power magnification (10 × 40-fold).
* Statistical difference with $P < 0.01$ (paired *t*-test).
† These stimulants contain 120 μl of PBS.

15 cases but not in OA-SF (Figure 1). We used the morphologic polarization assay to measure the activity of chemotaxis or its inhibition in the crude RA-SF. When we used the Boyden's chamber assay with crude RA-SF, an additional chemotactic activity was newly generated during the incubation period for 60 to 90 minutes and thereby the pre-existed inhibitory capacity to monocyte chemotaxis was artificially hidden. After purification of the chemotaxis inhibitors in RA-SF, we confirmed the activity using the multiwell chamber assay. Although correlation between the intensity of inhibitory activity and either the actual number or ratio of macrophages in RA-SF obtained was not clear, it is probably due to a presence of multiple factors to control the intensity of macrophage accumulation in RA-SF. For instance, the accumulation might be influenced by the area of blood vessel bed as well as the blood flow in the synovial tissue modified pathologically as pannus. It also must be influenced according to the disappearance rate of macrophages from the synovial cavity.

Table 6. *Cell-directed Inhibitory Effect of Low-Molecular-weight Chemotaxis Inhibitory Factor in RA-SF*

Stimulant	Pretreatment	Cells polarized(%) (mean ± SD) (n = 5)
ZAP	Inhibitor	18.8 ± 1.5 *
ZAP	PBS	39.3 ± 1.4 *
fMLP	Inhibitor	21.1 ± 2.5 *
fMLP	PBS	39.6 ± 1.5 *

Inhibitor, 120 μl of the pooled fraction in the gel permeation HPLC as shown in Figure 7 at the final concentration A_{280} = 0.006; PBS, 120 μl of phosphate-buffered saline.
* Statistical difference with $P < 0.01$ (paired *t*-test).
Inhibitory activity to monocyte chemotaxis was measured by the polarization assay as described in Table 2. Monocytes were pretreated with either the low-molecular-weight inhibitor obtained in the gel permeation HPLC or PBS for 30 minutes at 37°C, then washed twice with PBS using the polarization assay.
PBS, phosphate-buffered saline; ZAP, zymosan-activated plasma.

In RA-SF there were at least two inhibitory activities to monocyte chemotaxis, of which the molecular weights were 64 kd and 8 kd (Figures 2 and 3). The former had the same antigenicity as α_1-PI (Table 3) and possessed the effect on PMNs as well. Furthermore α_1-PI itself prepared from normal plasma had the inhibitory activity to the leukocyte chemotaxis in the morphologic polarization assay (Figures 4, 5). It was consistent with a previous report in which the inhibition of α_1-PI to PMN chemotaxis

Table 7. *Immunoadsorption of the Low-Molecular-weight Chemotaxis Inhibitory Factor by Anti-complement Antibodies*

Stimulant	Treatment	Cells polarized(%) (mean ± SD) (n = 5)
Experiment 1		
ZAP		49.7 ± 1.9
PBS		7.3 ± 2.9
ZAP + Inhibitor		12.9 ± 3.4
ZAP + Inhibitor	Anti-C3 antibody gel	13.0 ± 4.0 *
ZAP + Inhibitor	Anti-C4 antibody gel	49.6 ± 1.5 *
ZAP + Inhibitor	Anti-C5 antibody gel	14.7 ± 1.0
Experiment 2		
ZAP		41.8 ± 3.6
PBS		3.4 ± 1.0
ZAP + Inhibitor		21.0 ± 2.9
ZAP + Inhibitor	Anti-C4 antibody gel	39.9 ± 1.3 *

Inhibitor, the pooled fraction in the gel permeation HPLC as shown in Figure 7 (at the final concentration A_{280} = 0.006).
* Statistical difference with $P < 0.01$ (paired *t*-test).
Goat IgG antibodies against human complement component C3, C4, and C5 were immobilized on Sepharose 4B beads. In experiment 1 and experiment 2, anti-C4 antibodies obtained from Organon Teknica Co.-Cappel Products and from Behring-Werke Co. were used, respectively. Of the inhibitor fraction, 150 μl were treated with 150 μl of affinity gels at room temperature for 60 minutes with continuous shaking. After centrifugation at 10,000 rpm for 5 minutes, 120 μl of the supernatant was used in the polarization assay as Table 2. In the experiment without gels, 120 μl of the inhibitor fraction were used.

Table 8. *Generation of Similar Chemotaxis Inhibitor Activity in Plasma by Activation of Classical Complement Pathway*

Stimulant	Chamber assay (Migrated cell numbers at 5HPFs) (mean ± SD) (n = 5)	Polarization assay (Cells polarized(%)) (mean ± SD) (n = 5)
ZAP	673 ± 38	48.9 ± 0.8
PBS	8 ± 2	6.9 ± 1.0
ZAP + Sup A†	116 ± 29 *	11.2 ± 2.0 *
ZAP + Sup B†	660 ± 35 *	48.8 ± 0.8 *

Sup A, 120 μl of the supernatant fraction of 80% ammonium sulfate saturation of the aggregated IgG activated plasma (the classical pathway activation); Sup B, 120 μl of the supernatant fraction of 80% ammonium sulfate saturation of the zymosan activated plasma (the alternative pathway activation); 5HPFs, five fields selected at random in microscope observation with high-power magnification (10 × 40-fold).

* Statistical difference with $P < 0.01$ (paired *t*-test).

† Stimulant contains 40% v/v of either Sup A or Sup B.

Sup A and Sup B were used after dialyzed against PBS, as described in Table 4. Inhibitory activity to monocyte chemotaxis were measured by both the morphologic polarization assay and the chamber assay, as described in Table 2 and Table 5, respectively.

was demonstrated in Boyden's chamber assay.[21] It is well known that RA-SF contains plasma proteins due to an enhanced vascular permeability at synovial tissue.[20] According to the previous report by Borth,[24] the average concentration of α_1-PI in RA-SF is assumed to be 420 μg/ml. This concentration is enough to suppress the leukocyte chemotaxis at least *in vitro*. For these reasons, it was concluded that the high–molecular-weight inhibitor in RA-SF was α_1-PI itself or its derivative. Because it has been reported that the proteinase inhibitor from *Taenia taeniaeformis* and the synthetic inhibitors of serine protease such as L-1-tosylamide-2-phenylethyl-chloromethyl ketone and N-α-p-tosyl-L-lysine-chloromethyl-

Table 9. *Immunoadsorption of the Inhibitory Activity in Aggregated IgG-activated Plasma by Anti-complement Antibodies*

Stimulant	Treatment	Cells polarized(%) (mean ± SD) (n = 5)
ZAP		49.5 ± 3.6
PBS		6.9 ± 1.8
ZAP + Sup A		11.7 ± 4.1 *
ZAP + Sup A	Anti-C3 antibody gel	12.5 ± 4.0 *
ZAP + Sup A	Anti-C4 antibody gel	49.2 ± 3.0 *
ZAP + Sup A	Anti-C5 antibody gel	11.9 ± 4.0

Sup A, the supernatant fraction of 80% ammonium sulfate saturation of the aggregated IgG activated plasma.

* Statistical difference with $P < 0.01$ (paired *t*-test).

Goat IgG antibodies against human complement component C3, C4, and C5 were immobilized on Sepharose 4B beads. Sup A was dialyzed against PBS, as described in Table 4, before being used. Of Sup A, 150 μl were treated with 150 μl of affinity gels at room temperature for 60 minutes with continuous shaking. After centrifugation at 10,000 rpm for 5 minutes, 120 μl of the supernatant was used in the polarization assay, as in Table 2. In the experiment without the gels, 120 μl of Sup A were used.

ketone suppressed the chemotaxis of leukocytes,[21,25] it is probable that α_1-PI inhibits chemotaxis by means of the inhibition of a serine protease(s) that might be essential for the leukocyte chemotaxis. However α_1-PI may not be an important factor to establish the suppressed accumulation of macrophages in RA joint cavities because the inhibitor affected the PMN chemotaxis as well (Table 3).

On the other hand, the low–molecular-weight inhibitor only suppressed the chemotaxis of monocytes but not that of PMNs (Table 3). Furthermore, after removal of the low–molecular-weight inhibitor, a chemotactic ability to monocytes appeared in RA-SF (Table 4). Therefore the low–molecular-weight inhibitor may play a key role in causing the PMN-predominant infiltration in RA synovial cavities. There is a previous report that a C5a des Arg-like molecule, which was separated from RA-SF by a gel filtration column chromatography, possessed chemotactic activity to PMNs but not to monocytes.[3] This phenomenon probably was caused by the simultaneous presence of C5a des Arg and the low–molecular-weight inhibitor in the same fraction because pure C5a des Arg attracts chemotaxis not only to PMNs but also to monocytes,[8] and also because these two molecules could not be separated by gel filtration column chromatography because of their similar molecular size.

The pretreatment of monocytes with the low–molecular-weight inhibitor resulted in the marked diminution of the chemotactic response to ZAP as well as fMLP (Table 6). Therefore it is probable that the low–molecular-weight inhibitor is a cell-directed inhibitor in monocyte chemotaxis. Interestingly the low–molecular-weight inhibitor did not suppress the monocyte chemotaxis caused with PMA (Table 5). It is commonly thought that both ZAP and fMLP cause the leukocyte chemotaxis through the receptor-dependent signal transduction system in the cells.[26–30] On the other hand, it is believed that PMA causes its effect by the direct binding to and activation of protein kinase C with skipping of the step of receptor-dependent mediation.[31] For these reasons, the low–molecular-weight inhibitor is assumed to exhibit the inhibitory function by blocking a step(s) sometime after the ligand-receptor interaction but before the activation of protein kinase C in the signal transduction system of monocytes.

Because the low–molecular-weight inhibitor was absorbed markedly by the anti-C4 antibody affinity gels from two different sources (Table 7), and the similar inhibitor was generated in the plasma, of which the classical complement pathway but not the alternative pathway was activated, the inhibitor seems to be a breakdown product of the complement component C4 (Tables 8 and 9). It is well known that the activation of both the classical and alternative complement pathways is progressing in RA synovial cavities due to the presence of immune

complexes.[32–35] Thus it is probable that the inhibitor is generated on the occasion of the activation of the classical pathway of complement in RA-SF. In the ammonium sulfate fractionation and the CM-cellulose column chromatography, the low–molecular-weight inhibitor and an anaphylatoxin activity was cofractionated. Therefore we predicted that the inhibitor might be C4a or the derivative, although the anaphylatoxin activity disappeared during the gel filtration with the Toyopearl HW-50S. Because the anaphylatoxin activity of C4a was reported to be 2000 times less than that of C5a and, therefore, the minimum effective concentration of C4a in the guinea pig ileum contraction assay must be 10^{-6} mol/l,[36] the concentration in the fraction of the gel filtration chromatography might be outside the lowest limitation in the rat uterus contraction assay. For these reasons, the possibility that the low–molecular-weight size inhibitor is C4a or its derivative still remains. For identification of the C4-derived inhibitory factor, a high scale preparation to amino acid sequencing analysis is essential. Such a preparation from the complement-activated plasma is now in progress.

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References

1. Kambara T, Kutsuna T, Okamoto T, Nakamura T: Inflammatory activities of synovial fluid from rheumatoid and osteoarthritis to guinea pig skin. Acta Pathol Jpn 1982, 32:575–583
2. Ward PA, Zvaifler NJ: Complement derived leukotactic factors in inflammatory synovial fluid of humans. J Clin Invest 1971, 50:606–616
3. Okamoto T, Ueda K, Kambara T, and Kutsuna T: Chemotactic activity for polymorphonuclear and mononuclear leukocytes in rheumatoid synovial fluid. Acta Pathol Jpn 1986, 36:1109–1122
4. Klickstein LB, Shapleigh C, Goetzl EJ: Lipoxygenation of arachidonic acid as a source of polymorphonuclear leukocyte chemotactic factors in synovial fluid and tissue in rheumatoid arthritis and spondyloarthritis. J Clin Invest 1980, 66:1166–1170
5. Davidson EM, Rae SA: Leukotriene B_4, a mediator of inflammation present in synovial fluid in rheumatoid arthritis. Ann Rheum Dis 1983, 42:677–679
6. Snyderman R, Alfman LC, Hausman MS, Margenhagen SE: Human mononuclearleukocyte chemotaxis. A quantitative assay for humoral and cellular chemotactic factor. J Immunol 1972, 108:857–860
7. Snyderman R, Shin HS, Hausman MS: A chemotactic factor for mononuclear leukocytes. Proc Soc Exp Biol Med 1971, 138:387–390
8. Marder S R, Chenoweth DE, Goldstein IM, Perez HD: Chemotactic responses of human peripheral blood monocytes to the complement peptides C5a and C5a des Arg. J Immunol 1985, 134:3325–3331
9. Snyderman R, Pike MC: Structure and function of monocytes and macrophages. *In* McCarty DJ, ed. Arthritis and Allied Conditions 11th ed. Philadelphia, Lea and Febiger, 1989, pp 306–335
10. Gatter RA: Arthrocentesis technique and intrasynovial therapy. *In* McCarty DJ, ed. Arthritis and Allied Conditions, 11th ed. Philadelphia, Lea and Febiger, 1989, pp 646–656
11. Fernandez HN, Hugli TE: Partial characterization of human C5a anaphylatoxin. J Immunol 1976, 117:1688–1693
12. Hugli TE, Gerard C, Kawahara M, Scheetz ME, Barton R, Briggs S, Koppel G, Russel S: Isolation of three separate anaphylatoxins from complement-activated serum. Mol Cell Biochem 1981 41:59–66
13. Ouchterlony O: Diffusion in gel methods for immunological analysis. Prog Allergy 1953, 5:1–78
14. Böyum A: Isolation of mononuclear cells and granulocytes from human blood. Scand J Lab Invest 1968, 21(Suppl):77–89
15. Cianciolo GJ, Snynderman R: Monocyte resposiveness to chemotactic stimuli in a property of subpopulation of cells that can respond to multiple chemoattractants. J Clin Invest 1981, 67:60–68
16. Falk W, Goldwin RH, Leonard EJ: A 48 well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. J Immunol Method 1980, 33:239–247
17. Porath J, Axen RS, Ernbach S: Chemical coupling of proteins agarose. Nature 1967, 215:1491–1492
18. Cochrane CG, Müller-Eberhard HJ: The derivation of two distinct anaphylatoxin activities from the third and fifth components of human complement. J Exp Med 1968, 127:371–386
19. Arnette FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA Jr, Mithchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Huder GG: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988, 31:315–324
20. McCarty DJ: Synovial fluid. *In* McCarty DJ, ed. Arthritis and Allied Conditions, 11th ed. Philadelphia, Lea and Febiger, 1989, pp 69–90
21. Goetzl EJ: Modulation of human neutrophil polymorphonuclear leukocytes migration by human plasma alpha-globulin inhibitors and synthetic esterase inhibitors. Immunology 1975, 29:163–174
22. Perez HD, Hooper C, Volankis J, Ueda A: Specific inhibitor of complement (C5)-derived chemotactic activity in sys-

temic lupus erythematosus related antigenically to the Bb fragment of human factor B. J Immunol 1987, 139:484–489

23. Bianco C, Gotze O, Cohn ZA: Regulation of macrophage migration by products of the complement system. Proc Natl Acad Sci USA 1979, 76:888–891
24. Borth W, Dunky A, Kleesiek K: α_2-macroglobulin-proteinase complexes as correlated with α_1-proteinase inhibitor elastase complexes in synovial fluids of rheumatoid arthritis. Arthritis Rheum 1986, 29:319–325
25. Leid RW, Grant RF, Suquet CM: Inhibition of equine neutrophil chemotaxis and chemokinesis by a Taenia taeniaeformis proteinase inhibitor, Taeniaestain. Parasite Immunol 1987, 9:195–204
26. Chenoweth DE, Hugli TE: Demonstration of specific C5a receptor on intact human polymorphonuclear leukocytes. Proc Natl Acad Sci USA 1978, 75:3943–3947
27. Aswanikumar S, Corcoran B, Schiffmann E: Demonstration of a receptor on rabbit neutrophil for chemotactic peptides. Biochem Biophys Res Commun 1977, 4:810–817
28. Williams LT, Synderman R, Pike MC, Lefkowitz RJ:. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc Natl Acad Sci USA 1977, 74:1204–1206
29. Synderman R, Fudman EJ: Demonstration of chemotactic factor receptor on macrophages. J Immunol 1980, 124:2754–2757.
30. Spilberg I, Metha J: Demonstration of a specific neutrophil receptor for formyl-methionyl-leucyl-phenylalanine on the pulmonary alveolar macrophages and its relationship to chemotaxis and superoxide production. J Lab Clin Med 1981, 97:602–609
31. Niedl JE, Kuhn LJ, Vandenbark GR: Phorbol diester receptor couprifies with protein kinase C. Proc Natl Acad Sci USA 1983, 80:36–41
32. Pekin TJ, Zvaifler NJ: Hemolytic complement in synovial fluid. J Clin Invest 1964, 43:1372–1382
33. Hannestad K: Presence of aggregated γ-globulin in certain rheumatoid synovial effusions. Clin Exp Immunol 1967, 2:511–529
34. Ruddy S, Austen KF: The complement system in rheumatoid arthritis I. An analysis of complement component activities in rheumatoid synovial fluids. Arthritis Rheum 1970, 13:713–723
35. Ruddy S, Fearon DT, Austen KF: Depressed synovial fluid levels of properdin and properdin factor B in patients with rheumatoid arthritis. Arthritis Rheum 1975, 18:289–295
36. Gorski JP, Hugli TE, Müller-Eberhard HJ: C4a; The third anaphylatoxin of the human complement system. Proc Natl Acad Sci USA 1979, 76:5299–5302