The Chemical Mediation of Delayed Hypersensitivity Skin Reactions

III. Purification and Characterization of a Precursor Protein for Macrophage-Chemotactic Factor in Normal Guinea Pig Plasma

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A putative precursor protein for macrophage-chemotactic factor, which was extracted from inflammatory skin sites (MCFS-1) (Kambara et al, Am J Pathol 1977, 87:359–374), was found in normal guinea pig plasma and was purified to an apparent homogeneity upon SDS–polyacrylamide gel electrophoresis with a molecular weight of 160,000. This plasma protein was different from complement components of C3 and C5 in terms of molecular weight, functional activity as complements detected by hemolytic assay, and immunologic properties. Although it exhibited the common antigenicity with MCFS-1, it did not show any chemotactic activity for macrophages. However, incubation of this plasma protein at either 4 C for 5 days or 37 C for 1–2 days could generate a chemotactic factor with a molecular weight of approximately 150,000, which was similar to that of MCFS-1. This generation of chemotactic activity was completely prevented by the presence of the serine-type protease inhibitor, phenylmethylsulfonyl fluoride. These data could be well accounted for if we assume that this plasma protein might be a precursor for the macrophage-chemotactic factor found in delayed hypersensitivity skin sites, and that a proteolytic process might be involved in the activation of this precursor. (Am J Pathol 1982, 108:291–298)

WE HAVE SHOWN previously that in the skin sites of delayed hypersensitivity induced by bovine γ-globulin there occur three distinct macrophage-chemotactic factors in guinea pigs.[1,2] We named them as macrophage-chemotactic factor from skin (MCFS), MCFS-1, -2, and -3, respectively. The purification and the subsequent characterization of MCFS-1, a factor having the strongest chemotactic activity among these three factors, revealed that it was a heat-labile protein with a molecular weight of 150,000 and that it did indeed attract macrophages in the skin site when injected *in vivo*. More interestingly, this purified MCFS-1 gave a single precipitin line with anti-normal guinea pig serum antibody by immunodiffusion, which strongly suggested the presence of a substance sharing common antigenicity with MCFS-1 in normal guinea pig serum.

In order to find the mechanism of the development of delayed hypersensitivity skin reactions, we made an attempt to clarify the generating mechanism of MCFS-1, and special attention was paid to the nature of the antigenically equivalent serum protein to determine whether this serum protein was a precursor of MCFS-1 or not. We purified this serum protein from plasma, not from serum, to avoid any potential enzymatic cleavages during coagulation, and called this antigenically equivalent plasma protein to MCFS-1 "plasma factor." In this article, we report the purification and some properties of the plasma factor, which is antigenically equivalent to MCFS-1. The findings are consistent with the notion that the

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plasma factor might be converted to MCFS-1, with a concomitant generation of chemotactic activity via an enzymatic process, and that it is distinct from the fifth component of the complement.

Materials and Methods

Animals

Albino Hartley guinea pigs (300–500 g in body weight) were used.

Substances

DEAE-Sephadex A-50, CM-Sephadex, Sepharose 4B, and Sephadex G-50 and G-150 were purchased from Pharmacia Co., Uppsala, Sweden. Phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), L-lysine, polybrene, and benzamidine hydrochloride were obtained from Sigma, St. Louis, Missouri. Carrier ampholytes were products of LKB-Aminkemi, Bromma, Sweden. All other chemicals were reagent grade and were obtained both from Wako Pure Chemicals, Osaka, Japan, and from Nakarai Chemicals, Tokyo, Japan.

Plasma

Blood was collected by cardiac puncture with the use of 21-gauge needles and 20-ml polyethylene syringes, and a mixture of 9 parts of blood to 1 part of anticoagulant (Na_2 EDTA, 1.5%; glucose, 5%; and polybrene, 0.05%). The plasma was obtained by centrifugation at 2500 rpm for 20 minutes and then at 6000 rpm for 20 minutes and was kept frozen at −80 C until use.

Assay for Plasma Factor

Rabbit antiserum against MCFS-1 was prepared by three intradermal injections of purified MCFS-1 (500 μg, once per week) emulsified with an equal volume of complete Freund's adjuvant. The resulting antiserum gave a single precipitin line against normal guinea pig serum on immunoelectrophoresis as previously described[2] and was used to detect the plasma factor as an immunoreactive substance against anti-MCFS-1 antibody by immunodiffusion methods. It was carried out on 8 × 5 × 0.1 cm plastic plates (well, 3 mm in diameter; sample volume, 10–15 μl) coated with 1% agarose dissolved in 0.05 M barbital buffer, pH 8.6.

Estimation of Chemotactic Activity

We measured chemotactic activity for macrophages *in vitro* by Boyden's method with a modification described previously,[1,2] using a Nucleapore filter[3] (N500CPC 01300, Nucleapore Co., Pleasanton, Calif) and a blind well chamber made by clear acrylic plastic (343-0250, Bio-Rad Lab., Richmond, Calif; lower compartment, 100 μl, and upper compartment 200 μl). Oil-induced peritoneal macrophages (500 × 10^3 cells/ml) of guinea pig suspended in RPMI-1640 solution, pH 7.4, containing 0.5% bovine serum albumin (Sigma, St. Louis, Mo) were used. After incubation at 37 C for 90 minutes in moist air containing 5% CO_2, the macrophages that migrated to the lower surface of the filter were stained with Giemsa and counted over five randomly selected microscopic fields (10 × 40). The chemotactic activity was expressed as the mean number of macrophages determined in duplicate samples. Samples to be tested were dialyzed against phosphate-buffered saline (PBS) (pH 7.4) before use.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis

This was performed on 6% gels in 0.01 M phosphate buffer, pH 7.0, in the presence of 0.1% SDS as described by Weber and Osborn.[4] Before electrophoresis, samples were mixed with equal volumes of 0.02 M phosphate buffer, pH 7.0, containing 2% SDS, 8 M urea, and with or without 4% 2-mercaptoethanol, and were incubated at 100 C for 5 minutes. A drop of tracking dye (40% sucrose and 0.1% malachite green) was added to each sample, which was then subjected to electrophoresis on 6% polyacrylamide gels in 0.1% SDS. Electrophoresis was carried out of 8 mA/gel. The gels were stained with 0.125% Coomassie blue in methanol-water-acetic acid (50/40/10) mixture for 24 hours and destained with 7% acetic acid in 37 C water bath. The BDH molecular weight markers (range 56,000–280,000, Product No. 44230 2R, BDH Chemical Ltd., England) were used for molecular weight calibration, where the relative mobility of the proteins to the malachite green was plotted against the logarithm of the molecular weights.

Hemolytic Assay of C3 and C5

This was performed according to the method of Tack and Prahl.[5] The hemolytic activity of C3 was

measured with the use of $EAC1_{hu}4_{hu}$ cells (kindly given by the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) and R3 reagent depleted of C3 activity and, to lesser extent, of C4 and C5 activities. The C3 reagent was prepared by incubating fresh human serum with an equal volume of saturated KBr for 18 hours at 4 C. The KBr-treated serum was dialyzed against PBS, aliquoted, and stored at −70 C until use. To assay C3 activity, 0.4 ml of $EAC1_{hu}4_{hu}$ cells (1×10^8 cells/ml), 0.05 ml of 1 : 5 diluted R3 reagent, 0.01 ml of sample, and 0.54 ml of GVB^{2+} were incubated for 1 hour at 37 C. Cell lysis was stopped by the addition of 2.0 ml of ice-cold 0.15 M NaCl, each reaction mixture was centrifuged at 1800 rpm for 10 minutes to remove unlysed cells and cell debris, and the absorbance at 541 nm was measured. The hemolytic unit, CH_{50}, was defined as the reciprocal of the dilution of C3 that would lyse 1×10^7 cells.

The hemolytic activity of C5 was also measured using $EAC1_{hu}4_{hu}$ cells and an R5 reagent depleted of C3, C4, C2, and C5 activities. We prepared the R5 reagent by incubating fresh human serum with an equal volume of 1 M KSCN for 18 hours at 4 C and then further incubating it for 45 minutes at 37 C in the presence of 15 mM hydrazine. The serum thus obtained was dialyzed against PBS, aliquoted, and stored at −70 C. The C5 assay was performed in 0.4 ml of $EAC1_{hu}4_{hu}$ cells (1×10^8 cells/ml), 0.05 ml of 1 : 5 diluted R5 reagent, 0.01 ml of guinea pig C2 (1000 CH_{50} units/ml, Cordis Lab., Miami, Fla), 0.05 ml of guinea pig C3 (1000 CH_{50} units/ml), 0.01 ml of sample, and 0.48 ml of GVB^{2+}. The other methods were same as that described above in the C3 assay.

Results

Purification of the Plasma Factor

All procedures were performed at 4 C employing plastic or siliconized glass containers to avoid contact activation of Hageman factor. Unless otherwise specified, protease inhibitors (benzamidine, EDTA, and polybrene) were included in the purification buffers.

Step 1. DEAE-Sephadex Chromatography

Guinea pig plasma (500 ml) was dialyzed against 20 mM Tris-HCl buffer containing 40 mM NaCl, 3 mM EDTA, 50 mg/l polybrene, and 3.3 mM benzamidine (pH 8.0) for 15 hours. After removal of insoluble materials with centrifugation, the dialyzed sample was applied to a DEAE-Sephadex A-50 column (5 × 25 cm, bed volume 500 ml) equilibrated with the same buffer. Once the sample was loaded, the column was washed extensively with the starting buffer and developed with a linear NaCl gradient (total volume 8 l) in the same buffer to a limiting concentration of 300 mM NaCl. The flow rate was maintained at 125 ml/hr, each fraction (20 ml) was collected, and the absorbance at 280 nm was monitored. The elution profile in the gradient portion revealed a single but rather broad peak, and the plasma factor was eluted in the decrescent part of the peak corresponding in ionic strength to 160–200 mM NaCl (Figure 1A).

Step 2. Lysine-Sepharose Column Chromatography

The plasma-factor-rich fractions of the DEAE-Sephadex column were collected and dialyzed against 20 mM Tris-HCl buffer containing 40 mM NaCl, 3 mM EDTA, 50 mg/l polybrene, and 3.3 mM benzamidine (pH 8.0) for 16 hours. The sample thus dialyzed (about 1000 ml) was then applied to lysine-Sepharose 4B column (3 × 15 cm, bed volume 100 ml) equilibrated with the same buffer. The elution was accomplished by a linear NaCl gradient (300 ml of equilibration buffer and 300 ml of the same buffer containing 500 mM NaCl). Elution speed was approximately 50 ml/hr. The plasma-factor-rich fractions that appeared in the latter shoulder of the second peak (Figure 1B) were pooled. Diisopropyl fluorophosphate was immediately added to a final concentration of 1 mM. Since plasmin and plasminogen were eluted in the buffer containing 200 mM ε-amino-n-caproic acid (data not shown), the adsorption of the plasma factor to this column might be due to the weak ion-exchange properties of lysine-Sepharose 4B.[6]

Step 3. CM-Sephadex Chromatography

The pooled fraction (250 ml) at step 2 was dialyzed against 20 mM phosphate buffer containing 40 mM NaCl and 0.5 mM PMSF (pH 6.6) for 8 hours and was applied to a column (2 × 16 cm, bed volume 50 ml) of CM-Sephadex previously equilibrated with the above buffer. A linear gradient elution with NaCl up to 300 mM in 20 mM phosphate buffer (pH 6.6) yielded two major components (Figure 1C). The plasma factor was exclusively concentrated in the second one.

Step 4. Isoelectric Focusing (IEF) Fractionation

The plasma factor (3–5 mg) was dialyzed against 20 mM phosphate buffer containing 40 mM NaCl and

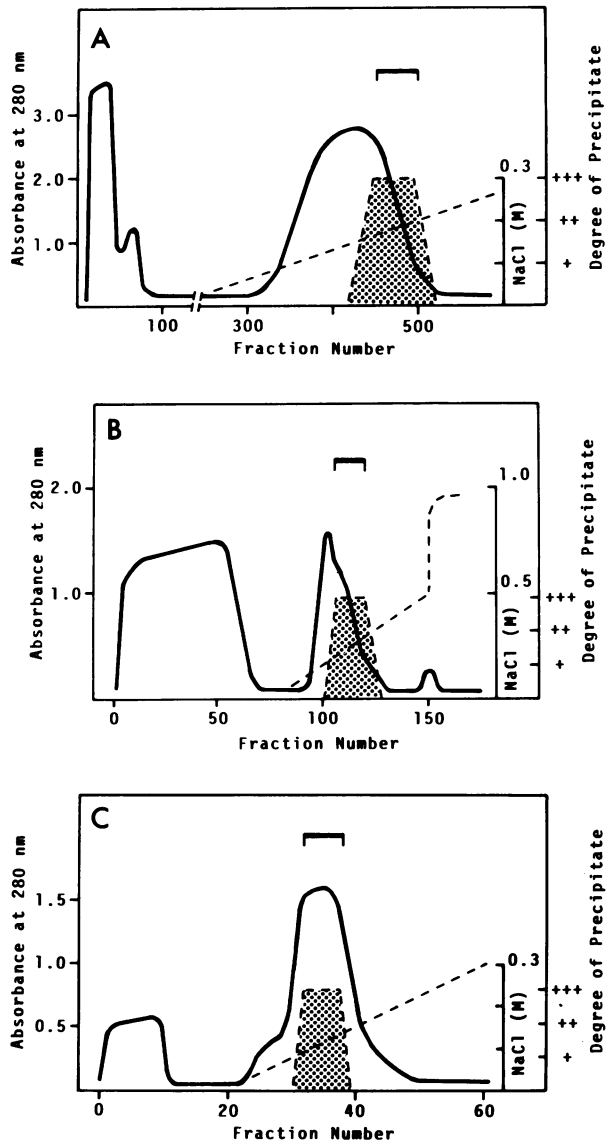

Figure 1A—DEAE-Sephadex chromatography of the plasma factor. Fractions were collected every 20 ml. **B**—Lysine-Sepharose chromatography of post-DEAE-Sephadex fractions. Fractions were collected every 20 ml on the break-through portion and every 10 ml on the gradient portion. **C**—CM-Sephadex C-50 chromatography of post-Lysine-Sepharose fraction. Fractions were collected every 20 ml on the break-through portion and every 10 ml on the gradient portion. The *dotted area* represents the plasma factor activity (degree of precipitate, + ∿ + + +); the *solid line* represents the absorbance at 280 nm; the *broken line* represents the NaCl concentration. Fractions indicated by the bar were pooled for the next purification step. For details, see text.

0.5 mM PMSF for 6 hours and was applied to an electrofocusing column (110 ml volume, Kato Shoten, Osaka, Japan). Focusing was performed with the use of carrier ampholyte (final concentration 1%, mixture of pH 3.5-10 [1 part] and pH 5-8 [4 parts]) and a sucrose gradient. After focusing for 40 hours at a constant voltage, 600-700 V, 1.5-ml serial fractions were collected from the bottom of the column. The pH of each fraction was measured immediately at 4 C. As shown in Figure 2, the plasma factor was concentrated on the fractions at pH 5.4 ± 0.1 (mean value ± SD for triplicate experiments). On the other hand, two independent methods were used to estimate the position of C5 and C3. One was the hemolytic assay of complement as described in Materials and Methods, and the other was an immunologic analysis by using both anti-guinea pig C5 antibody (kindly donated from Dr. H. S. Shin, The Johns Hopkins University, Baltimore, Md) and anti-guinea pig C3 antibody prepared in our laboratory. The results obtained from these two separate approaches agreed well. They showed pI values for C3 and C5 of 6.8 ± 0.1 and 5.4 ± 0.2, respectively, suggesting that the plasma factor was different from C5 in their pI values. The plasma factor-rich fractions were pooled and passed through a Sephadex G-50 column (1.6 × 15 cm, bed volume 30 ml) equilibrated with phosphate-buffered saline (pH 7.4) to remove ampholytes.

Some Properties of the Plasma Factor

When the purified plasma factor was subjected to SDS-polyacrylamide gel electrophoresis, a single protein band was observed either in the presence or absence of the reducing reagent (Figure 3), indicating that the plasma factor was composed of a single polypeptide chain with a molecular weight of 160,000, as estimated by calibration with the standard marker proteins.

On immunodiffusion, the purified plasma factor gave a single precipitin line with anti-guinea pig serum antibody (anti-GPS) and anti-MCFS-1 antibody (data not shown). As shown in Figure 4, anti-MCFS-1

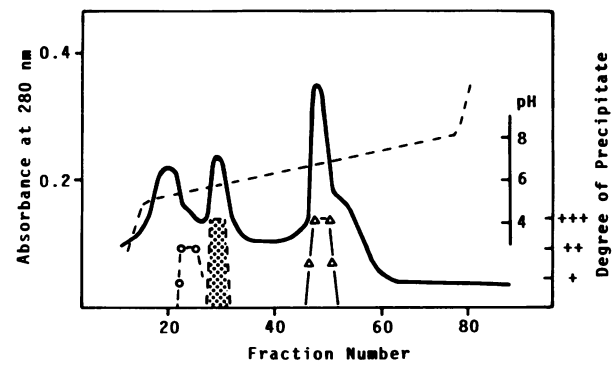

Figure 2—Isoelectric focusing of the plasma factor after CM-Sephadex. Fractions were collected every 1.5 ml, and the pH was measured immediately at 4 C. The *dotted area* represents the plasma factor activity; *open circles* represent C5 activity (degree of precipitate by specific antibody and complement hemolytic activity); *open triangles* represent C3 activity; the *thin broken line* represents the pH gradient; the *solid line* represents the absorbance at 280 nm.

antibody gave a single precipitin line with both MCFS-1 and plasma factor, and these lines joined each other without any spur formation. In order to determine whether or not the plasma factor was different from C5, we immunodiffused the plasma factor both against anti-MCFS-1 antibody and against anti-guinea pig C5 antibody. The analysis of the specificity of the anti-guinea pig C5 antibody (donated from Dr. H. S. Shin) done in our laboratory revealed that it gave a single precipitin line against normal guinea pig serum on immunodiffusion and could also deplete the hemolytic activity of C5 on hemolytic assay. When highly purified plasma factor, although containing C5, was poured into the upper well and anti-MCFS-1 antibody and anti-guinea pig C5 antibody were poured into each of the lower ones, two precipitin lines were found, but no interaction was recorded between them (Figure 5).

Furthermore, we analyzed the purified plasma factor to determine whether it contained hemolytic activity of C5 and C3. The result showed that the plasma factor preparation (peak fractions on IEF) contained neither C5 nor C3 hemolytic activities (Table 1). Thus, based on these immunologic and functional criteria, we concluded that the plasma factor was neither complement component nor its derivative of C5 or C3.

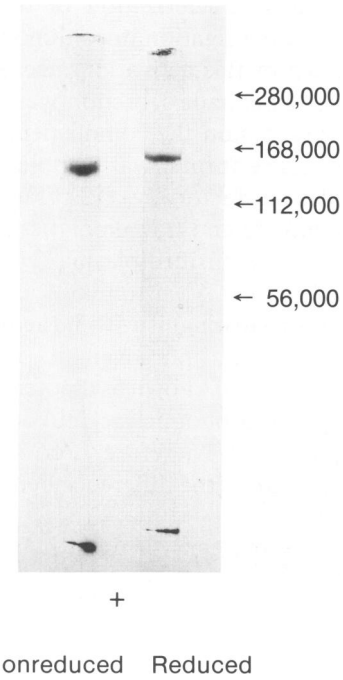

Figure 3—SDS-polyacrylamide gel electrophoresis of the purified plasma factor. The polypeptide structure of the post-IEF plasma factor was shown by polyacrylamide gel electrophoresis (6%) in 0.1% sodium dodecyl sulfate. The samples shown were any prior reduction (*left*) and with prior reduction by 2-mercaptoethanol (*right*). The molecular weights shown on the right side are those of the marker proteins.

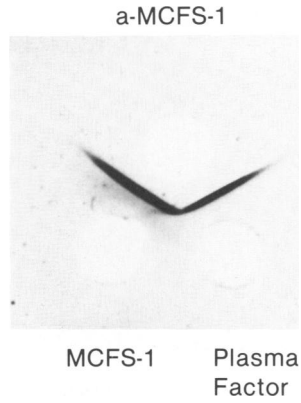

Figure 4—Immunodiffusion of the plasma factor. Upper well, anti-MCFS-1 antibody; lower left, MCFS-1 (1.5 mg/ml); lower right, plasma factor (1.0 mg/ml).

Estimation of Chemotactic Activity of the Plasma Factor

The post-CM-Sephadex fraction and post-IEF fraction were dialyzed against PBS (pH 7.4) and tested for their macrophage-chemotactic activity. As shown in Table 2, the two plasma factor preparations revealed negligible, if any, chemotactic activity. MCFS-1, which was purified from delayed hypersensitivity skin sites, however, exhibited strong activity even at the lower protein concentration.

Generation of Chemotactic Activity From the Plasma Factor

Although the plasma factor exhibited same antigenicity with MCFS-1, it was completely devoid of any chemotactic activity, strongly suggesting that the plasma factor could be a putative precursor for MCFS-1. To test this possibility, the conversion of the plasma factor to MCFS-1 in parallel with a generation of chemotactic activity was examined. We found that the incubation of the plasma factor in PBS (pH 7.4) resulted in an appearance of chemotactic activity for 4–5 days at 4 C or 24–48 hours at 37 C only in the absence of phenylmethylsulfonyl fluoride. Since PMSF prevented it from activation, as shown in Table 3, it is relevant for us to think that some enzymatic processing (limited proteolysis) might be involved in this activation process.

The appearance of chemotactic activity was also observed with the plasma factor from post-CM-Sephadex preparation when incubated for 8–12 hours at 37 C without PMSF. We fractionated this activated

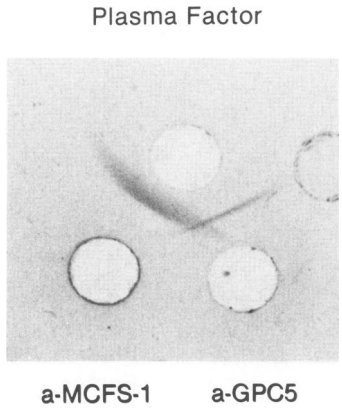

Figure 5—Immunodiffusion of the partially purified plasma factor. Upper well, unpurified plasma factor (post-CM-Sephadex preparation also containing C5 and C3); lower left, anti-MCFS-1 antibody; lower right, anti-guinea pig C5 antibody.

sample by gel filtration on Sephadex G-150 column to estimate the molecular weight of the generated chemotactic factor. The column was equilibrated with PBS (pH 7.4), and the sample (1.8 ml) was loaded. The gel filtration pattern was shown in Figure 6. The macrophage-chemotactic activity emerged slightly after the void volume, and none of the chemotactic activity was found in the low-molecular-weight portion. Its molecular weight was estimated approximately 150,000 by calibration with standard proteins, and was indistinguishable from that of MCFS-1.[1]

Discussion

In the present article we have described purification and characterization of a precursor protein in plasma (plasma factor) for the macrophage-chemotactic factor from inflammatory skin sites (MCFS-1). This plasma factor shares common antigenicity with MCFS-1, which shows the strongest chemotactic activity among three factors (MCFS-1, -2 and -3) found in delayed hypersensitivity skin reaction sites induced by bovine γ-globulin in guinea pigs.[1,2] The plasma factor was purified with the use of DEAE-Sephadex, lysine-Sepharose, CM-Sephadex, and isoelectric focusing, in this order, and the purified material was homogeneous, with a single polypeptide chain, upon SDS-polyacrylamide gel electrophoresis (Figure 3). Its molecular weight was determined to be 160,000; and it was almost same or slightly larger than MCFS-1 (mol wt 150,000 by gel filtration on Sephadex G-200[1]). During this purification procedure, the following precautions were made with regard to the suppression of the activation of endogeneous proteolytic enzymes present in the blood: 1) benzamidine, EDTA and PMSF were added in the buffer used during the chromatographies; 2) the pooled fractions were treated with DFP in each purification step; 3) polybrene was added in the buffer, and we used plastic or siliconized glass containers to avoid the activation of Hageman factor, which was reported to cause the activation of other proteolytic enzymes; 4) lysine-sepharose affinity chromatography was used to remove plasmin and plasminogen.

The plasma factor thus purified possessed no chemotactic activity in itself (Table 2), but a significant chemotactic activity was generated from the plasma factor by incubation for 1–2 days at 37 C or 4–5 days at 4 C in the absence of protease inhibitor (Table 3). Since this activation had not taken place in the presence of PMSF (0.5 mM), some enzymatic proteolysis seemed to be responsible for the appearance of chemotactic activity in the plasma factor fraction. Two possibilities should be taken into consideration that could explain this phenomenon. First, the plasma factor itself possesses a proteolytic activity which gives rise to the generation of chemotactic activity via autolytic cleavage. Second, the proteolytic enzyme(s) which is involved in this conversion mechanism is contaminated in this preparation. Although data is not published here, our recent isolation of a trace but significant amount of PMSF-sensitive enzyme activity from the purified plasma factor by the use of benzamidine-cellulose affinity column favors the latter possibility. The characterization of this enzyme is now under our investigation in conjunction with further elucidation of this activation mechanism.

The post-CM-Sephadex fraction was immunologically shown to contain the complement components of C3 and C5. The former was completely separated from the plasma factor by IEF fractionation, as shown in Figure 2, and no hemolytic activity of C3 was detected in the purified plasma factor (Table 1). In a practical sense, C5 was one of the most difficult serum proteins to distinguish from the plasma factor in purification; and, moreover, its anaphylatoxin fragment, C5a, is well known as a potent chemotactic factor for polymorphonuclear leukocytes (PMNs) and macrophages.[7-9] Therefore, a critical evaluation was needed to clarify the difference between the plas-

Table 1—Analysis of Hemolytic Activity of C3 and C5 in the Plasma Factor

Sample	C3 activity (CH_{50} units/ml*)	C5 activity (CH_{50} units/ml*)
Purified plasma factor†	< 1	< 1
Normal guinea pig plasma	15.4×10^3	41.5×10^3

* The hemolytic unit, CH_{50}, was expressed as the reciprocal of the dilution that would lyse 1×10^7 cells as described in Materials and Methods.

† Purified plasma factor used was the peak fraction on isoelectric focusing fractionation.

ma factor and the complement component of C5. Our data presented here support the contention that the plasma factor is evidently different from C5 for the following reasons: 1) Upon the isoelectric focusing, C5 exhibited a slightly but distinctly lower pI value than the plasma factor (Figure 2). 2) The purified plasma factor was revealed to be a single polypeptide chain with a molecular weight of 160,000 upon SDS-polyacrylamide gel electrophoresis (Figure 3). C5 has been demonstrated, however, to be composed of two nonidentical polypeptide chains of molecular weights of 115,000 and 75,000, respectively, which are linked via disulfide bridges.[10] 3) The immunodiffusion experiment on the plasma factor done both against anti-MCFS-1 antibody and against anti-guinea pig C5 antibody showed that they were immunologically distinct from each other (Figure 5). 4) The molecular weight of C5a was reported to be approximately 15,000, whereas that of the chemotactic factor generated from the plasma factor was determined to be approximately 150,000 (Figure 6). Taken together, the above findings strongly suggest that the plasma factor purified from guinea pig plasma might belong to an entity different from the complement component of C5. However, we cannot exclude the possibility at present, on the basis of the molecular weight, of the incomplete cleavage of C5, the complex formation of C5a with high-molecular weight-protein, the aggregation of C5a itself, or the complex formation of C5a with some cleavage of C5. However, we have not yet encountered any reports of a chemotactic factor derived from C5 the molecular weight of which was 150,000.

Recently, Fernandez et al[13] and Perez et al[14,15] reported "helper factor" or "cochemotaxin" existing in normal human serum. This factor permitted low concentrations of C5a des Arg to exhibit significant chemotactic activity, and was a heat-stable (56 C for 30 minutes) anionic polypeptide (approximately 60,000

Table 2—Macrophage Chemotactic Activities in Plasma Factor Fractions

Sample*	Protein concentration†	Chemotactic activity‡
Plasma factor (post-CM-Sephadex)	0.10	8
Plasma factor (post-IEF)	0.10	4
MCFS-1	0.08	130
Buffer§	–	4

* All samples were stored at −70 C containing 0.5 mM PMSF until use, and dialyzed against PBS (pH 7.4) before chemotactic assay.

† Protein concentration was measured by an absorbance at 280 nm.

† Chemotactic activities were expressed as the mean number of migrated macrophages per 5 high-power fields.

§ Phosphate-buffered saline, pH 7.4.

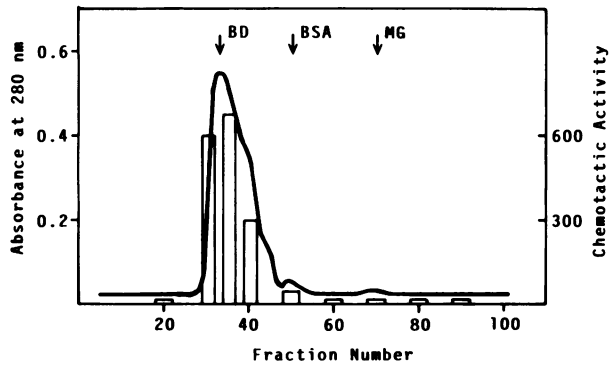

Figure 6—Gel filtration of the activated plasma factor on Sephadex G-150. The plasma factor preparation obtained after CM-Sephadex chromatography was incubated for 8 hours and then applied on the column (1.8 × 55 cm, bed volume 140 ml) of Sephadex G-150 equilibrated with phosphate-buffered saline (pH 7.4). Fractions were collected every 1.6 ml, with a flow rate of 8 ml/hr. The *solid line* represents the absorbance at 280 nm; *open columns* represent the chemotactic activity (numbers of migrated macrophages per 5 fields). Blue dextran (*BD*), bovine serum albumin (*BSA*, 67,000 in molecular weight), and myoglobin from horse heart (*MG*, 18,800 in molecular weight) were used as markers.

in molecular weight).[15] Although both C5a des Arg and cochemotaxin are reported to be heat-stable, MCFS-1 was a heat-labile protein[1]; and the chemotactic activity generated from the plasma factor in the present study seemed to be different from their molecular weight analysis.

Several authors have observed chemotactic activity in serum,[16-20] and we also found macrophage-chemotactic activity in normal guinea pig serum, the molecular weight of which was similar to that of IgG (unpublished data). Snyderman et al[16] described the existence in mouse serum of two chemotactic factors, of low molecular weight and of high molecular weight. The former was C5a, and the latter was clearly absent in C5-deficient serum, indicating that C5 was required for their presence. On the other hand, Jungi[19] found monocyte-chemotactic activity in diluted human serum and plasma, which seemed to be unrelated to the recognized component of complement, kallikrein formation, clotting, fibrinolysis, or isoantibody-blood-cell-component interaction. Many of these reported chemotactic factors present in serum seem to be different from MCFS-1 because of the difference in their molecular weight. It is not possible to go beyond this statement, however, without any satisfactorily purified preparations of the high-molecular-weight chemotactic factors in serum.

In the present paper, we reported the precursor present in normal guinea pig plasma for the macrophage-chemotactic factor (MCFS-1) and the involvement of a proteolytic mechanism in the conversion of the plasma factor to a chemotactically active substance. The molecular weight of the generated che-

Table 3—Appearance of Chemotactic Activity from the Plasma Factor*

Incubation time	PMSF added†	Chemotactic activity‡
No treatment	–	14
	+	9
3 days at 4 C	–	10
	+	8
5 days at 4 C	–	220
	+	10
48 hours at 37 C	–	267
	+	11

* Post-IEF plasma factor was used, and the protein concentration was 0.15 (absorbance at 280 nm).

† PMSF used was 0.5 mM in a final concentration, and all the samples were dialyzed against PBS (pH 7.4) before chemotactic assay.

‡ Results of a typical experiment.

motactic substance from the plasma factor was approximately 150,000 and was quite similar to that of MCFS-1. Although further studies on the activation mechanism of the plasma factor are necessary, it is relevant from the present study that the plasma factor might be a precursor form of MCFS-1 found in delayed hypersensitivity skin sites *in vivo*. The mechanism of this conversion might give us an important key to elucidate the initiation of macrophage migration into the skin site of delayed hypersensitivity *in vivo*, and the nature of PMSF-sensitive protease remains to be determined.

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