A Chemotactic Factor for Mononuclear Leukocytes[1] (35903)

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(Introduced by Dr. S. E. Mergenhagen)

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There is mounting evidence to suggest that the complement (C) system plays an important role as an effector of the acute inflammatory response (1–3). In particular, cleavage of the fifth component of C results in the release of a product, C5a, which is able to mediate a number of important biological activities. C5a has been shown to increase vascular permeability, contract certain types of smooth muscle and chemotactically attract polymorphonuclear leukocytes (PMN) (4–9). These biological activities are prominent events in inflammatory states such as the local Arthus reaction, a model of immunologically induced tissue damage. Another important event in the acute inflammatory process is the local accumulation of mononuclear leukocytes (MNL). These cells appear within a few hours of the onset of the inflammatory event and perform the critical function of phagocytizing degenerating PMN and their fragments, as well as tissue debris (10). Moreover, accumulation of MNL locally appears to play an essential role in subsequent wound healing (11). Since C5a can mediate several of the biological events initiated by local antigen–antibody reactions and since the MNL plays an important role in the pathophysiology of such reactions, experiments were performed to determine if C5a affected the migration of MNL *in vitro*.

Materials and Methods. Highly purified guinea pig C3 and C5 were prepared by methods previously described (12, 13). Crystallized trypsin and soybean trypsin inhibitor was obtained from Worthington Biochemicals, Freehold, N.J. The products cleaved from C5 by sensitized sheep erythrocytes carrying C1, C4, and C2, and C3 ($EAC\bar{1},\bar{4},\bar{2},\bar{3}$ were prepared as described (9)). Chemotaxis of PMN or MNL was quantitated *in vitro* using a modification of the method of Boyden (14). PMN were obtained from the peritoneal cavity of rabbits or guinea pigs which had been stimulated with glycogen 16 hr before (15). MNL-rich exudates were obtained from the peritoneal cavity of guinea pigs which had been stimulated with 0.5% shellfish glycogen 4 days before. The latter exudate cells contained at least 80% large MNL, with the remainder of the cells being composed of either PMN or small lymphocytes. In experiments with MNL, 8.0 μ Micropore filters (Brinkman Corporation, Westbury, NY) were used in the chemotaxis chambers.

Results. In the first experiment, C3 or C5 was treated with trypsin at 25° for a period of time ranging from 10 to 60 min. The reaction was stopped by the addition of soybean trypsin inhibitor. The resultant reaction mixtures were diluted in Gey's balanced salt solution (15) and tested for PMN as well as MNL chemotatic activity. Hemolytic C assays for C3 and C5 (16, 17) were performed on each reaction mixture to determine the percentage of C3 and C5 destroyed by trypsin. Part A of Table I shows that solutions containing 2.8 μg of C5/ml, which had been treated with trypsin, were strongly chemotactic for PMN. In this experiment, approximately 30% of the hemolytic activity of C5 was destroyed by trypsin. C5a is approximately 10% of C5 by molecular weight and since only 30% of the C5 available was cleaved in this experiment, the amount of C5a needed to give a strong chemotactic re-

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TABLE I. Chemotactic Activity of Trypsinized C3 and C5 for Rabbit Polymorphonuclear Leukocytes and Guinea Pig Mononuclear Leukocytes.

Reaction mixture	Chemotactic activity[a] Expt: 1	2
A. Rabbit polymorphonuclear leukocytes		
C3[b] (34.0 μg) + trypsin	0	4
C3 (5.6 μg) + trypsin	0	—
C3 (1.2 μg) + trypsin	0	—
C5[c] (17.0 μg) + trypsin	216	264
C5 (2.8 μg) + trypsin	216	228
C5 (0.6 μg) + trypsin	—[d]	24
C3 (34.0 μg) solo	0	—
C5 (17.0 μg) solo	12	4
B. Guinea pig mononuclear leukocytes		
C3[b] (10 μg) + trypsin	6	21
C5[c] (5 μg) + trypsin	106	67
C3 (10 μg) solo	9	6
C5 (5 μg) solo	8	11

[a] Expressed as cells per high power field.

[b] C3 (1 mg) was reacted (25° for 30 min) with trypsin (10 μg) followed by the addition of soybean trypsin inhibitor (20 μg). Approximately 60% of the hemolytic activity of C3 was destroyed by this reaction. The reaction mixture was then serially diluted in Gey's medium and the dilutions originally containing the indicated amounts of C3 were tested for chemotactic activity.

[c] C5 (500 μg) was reacted (25° for 30 min) with trypsin (5 μg) followed by the addition of soybean trypsin inhibitor (10 μg). Approximately 30% of the hemolytic activity was destroyed by this reaction. The reaction mixture was then serially diluted in Gey's medium and dilutions originally containing the indicated amounts of C5 tested for chemotactic activity.

[d] Not tested.

sponse was 85 ng/ml. In other experiments, solutions containing approximately 50 ng of C5a/ml were also strongly chemotatic for PMN. Similar findings were obtained when guinea pig PMN were substituted for rabbit PMN. On the other hand, in no experiment was chemotactic activity for PMN detected by cleavage of guinea pig C3 with trypsin, even when C3 was used in amounts 100 times in excess of C5 needed for the detection of chemotactic activity.

In parallel experiments, C3 or C5 was cleaved with trypsin, and then tested for chemotactic activity using guinea pig MNL (Part B of Table I). Marked chemotactic activity for MNL was found in reaction mixtures containing C5 cleaved by trypsin with the maximum amount of C5a/ml being 150 ng. In no experiment was MNL chemotactic activity found in solutions containing different quantities of C3 cleaved by trypsin for varying time periods.

In addition to trypsinization, C5 was also cleaved by the earlier acting C components and tested for MNL chemotactic activity. As shown in Table II, supernatants of EAC$\overline{1}$,$\overline{4}$,2, $\overline{3}$ were not chemotactic for MNL. On the other hand, the addition of C5 to EAC$\overline{1}$,$\overline{4}$,2, $\overline{3}$, resulted in the generation of MNL chemotactic activity in the supernatants of this reaction mixture. Doubling the concentration of supernatant tested approximately doubled the amount of MNL chemotactic activity detected. The preceding experiments demonstrated that cleavage of C5 by a proteolytic enzyme, as well as by the earlier acting C components resulted in the production of MNL chemotactic activity.

In order to characterize the MNL chemotactic factor, C5 (500 μg) was cleaved with trypsin (30 min at 25°) followed by the addition of soybean trypsin inhibitor. This reaction results in the destruction of approximately 40% of the C5 hemolytic activity. A

TABLE II. Chemotactic Activity of C5 Cleaved by $EAC\overline{1},\overline{4},\overline{2},\overline{3}$ for Mononuclear Leukocytes.

Reaction mixture[a]	Chemotactic activity
$EAC\overline{1},\overline{4},\overline{2},\overline{3}$ + buffer	0
+ C5 (3.5 mg)[b]	36
+ C5 (7.0 μg)[b]	64
C5 solo (7.5 μg)	1

[a] C5 was cleaved by $EAC\overline{1},\overline{4},\overline{2},\overline{3}$ at 37° in GVB^{2+} diluted with an equal volume of 5% dextrose in water. Incubation was carried out for 5 min at a final concentration of 500 μg/ml and $EAC\overline{1},\overline{4},\overline{2},\overline{3}$ concentration of 1.5×10^{10} cells/ml.

[b] Amount indicated represents original input of C5 before cleavage.

portion of the reaction mixture, containing 250 μg of C5 was chromatographed on a Sephadex G-75 column equilibrated with pH 7.2 phosphate buffered (0.02 *M*) isotonic saline (PBS). Cytochrome *c* (12,500 mol wt) was chromatographed separately as a molecular weight marker. The chemotactic activity fractions was tested using either PMN or MNL. A single peak of chemotactic activity for both PMN and MNL was found to emerge just prior to the cytochrome *c* marker (Fig. 1). The molecular weight of these activities was calculated to be approximately 15,000 (18). This value agrees with previous determinations of the molecular weight of C5a where C5 was cleaved by earlier acting C components (9).

In the last experiment, C5 cleaved by $EAC\overline{1},\overline{4},\overline{2},\overline{3}$, was fractionated by polyacrylamide gel electrophoresis at acid pH (19). Following electrophoresis, gels were cut into segments and eluted with gelatin–Veronal buffer, pH 7.3 (GVB^{2+}) (20). The eluates were tested for PMN as well as MNL chemotactic activities. A single segment contained both PMN and MNL chemotactic activities (Fig. 2). In contrast, similar fractionation of the reaction mixture of $EAC\overline{1},\overline{4},\overline{2},\overline{3}$, without C5 produced no chemotactic activity.

Discussion. These experiments suggest that a single cleavage product of C5, whether obtained as a result of reaction of C5 with a proteolytic enzyme or $EAC\overline{1},\overline{4},\overline{2},\overline{3}$, contains chemotactic activity for both PMN as well as

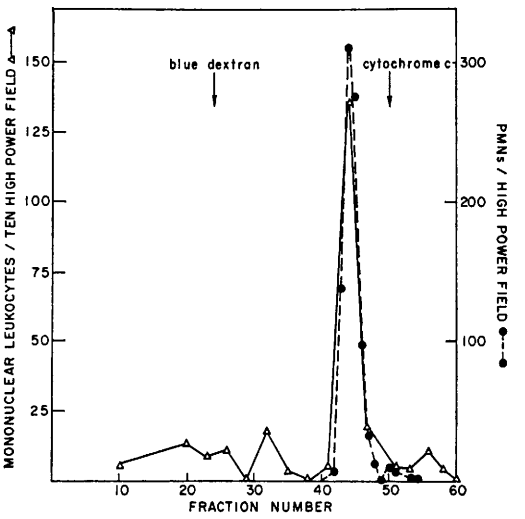

FIG. 1. Characterization of the chemotactic activity of trypsin-treated C5 by gel filtration. C5 (500 μg) was reacted (25° for 30 min in PBS) with trypsin (5 μg) followed by the addition of soybean trypsin inhibitor (10 μg). A portion of this reaction mixture containing 250 μg of C5 was added to a Sephadex G-75 column (0.75 × 60 cm) and eluted with PBS. Fractions were tested for either PMN or MNL chemotactic activity. Blue dextran (mol wt 2,000,000) and cytochrome *c* (mol wt 12,500) were used as molecular weight markers and chromatographed independently of the C5 reaction mixture.

MNL. It is now clear that C5a effects a number of biological activities which are important not only in the mediation of acute inflammation, but also in wound healing. The PMN appear to play an important role in the early phase of acute inflammation by phagocytizing immune complexes or bacteria. While the PMN dominate the histological picture early in the inflammatory reaction, the MNL appear shortly thereafter and dominate the later aspects of the reaction participating both in the removal of PMN debris and the early phases of wound repair (11).

While factors responsible for the accumulation of MNL at sites of delayed hypersensitivity have been previously described (21), our findings illustrate a mechanism by which MNL might accumulate under conditions where the cleavage product of C5 would be found. Such conditions would include local antigen–antibody reactions, nonspecific tissue

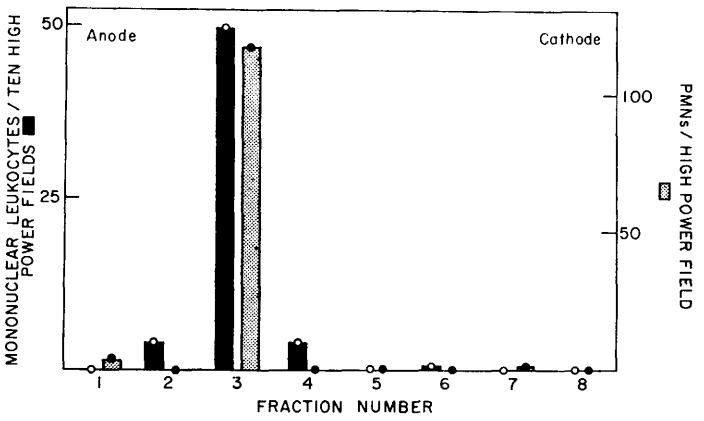

FIG. 2. Polyacrylamide-gel electrophoresis of C5 reacted with EAC$\overline{1,4,2,3}$. C5 was derived by EAC$\overline{1,4,2,3}$ at the C5 concentration of 500 μg/ml. Following centrifugation to remove the cells, the supernatant was acidified to pH 3.5 with 1.0 *M* acetic acid as described in (9). About 0.05 ml of supernatant was fractionated by polyacrylamide gel disc electrophoresis at low pH (19). The concentration of acrylamide in the small pore gel (0.8 × 4.5 cm) was 7.5%. Electrophoresis was carried out at 4–6° for 3 hr at 50 V. The small pore gel was cut into 8 segments and each segment was eluted in 1 ml of GVB^{2+}.

trauma, and local cytolytic virus infections (22).

Summary. Chemotactic activity for mononuclear leukocytes was produced when highly purified guinea pig C5 was cleaved by trypsin or by the earlier acting complement components. This chemotactic activity was mediated by C5a, a fragmentation product of the fifth component of complement. These findings suggest a new biological function for the complement system, *i.e.*, chemotaxis of mononuclear leukocytes, cells which are important in acute inflammation as well as wound healing.

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