CHEMOTACTIC ACTIVITY GENERATED FROM THE FIFTH COMPONENT OF COMPLEMENT BY PLASMA KALLIKREIN OF THE RABBIT*

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The proteins of the complement and coagulation systems are major transducing mechanisms of plasma whereby small initiating signals are amplified and translated into biological events. To date, these systems have largely been studied in isolation, although interactions between them may be expected. Indeed the similarity of structure and mechanism of inhibition of Cls and Clr of the complement system (1) and Hageman factor, Factor XI, and prekallikrein of the intrinsic coagulation system (2) are so similar as to raise the possibility of genetic reduplication in their development. Furthermore, plasma kallikrein has been shown to be capable of cleaving and activating both Cls and Clr (3).

In the present study, we report the ability of kallikrein purified from rabbit plasma to generate chemotactic activity for rabbit neutrophils from rabbit C5. The study was prompted by previous reports that human kallikrein was directly chemotactic for human neutrophils (4), an observation that we have not been able to confirm for the rabbit system. The results suggest the existence of a novel interaction between the Hageman factor and complement systems which may have biological relevance.

Materials and Methods

Purified Proteins from Rabbit Plasma

HAGEMAN FACTOR (HF).[1] HF and the two-chain, 80,000-M_r form of activated HF (α-HFa) were purified from rabbit plasma (Fig. 1).[2]

PREKALLIKREIN (PK). PK was purified from citrated rabbit plasma in collaboration with Dr. William McGuire of the Research Institute of Scripps Clinic, La Jolla, Calif., by a three-step procedure.

Step 1. Solid-Phase Immunoabsorption of PK from Rabbit Plasma. An IgG fraction of monospecific

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; Bz-pro-phe-arg-pNA, *N*-Benzoyl-L-Pro-L-phe-L-arginine-*p*-nitroanalide; HBSS, Hanks' balanced salt solution; HF, Hageman factor; HFa, activated HF; α-HFa, the 80,000-M_r form of HFa; LDH, lactic dehydrogenase; PK, prekallikrein; SBTI, soybean trypsin inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ZARS, zymosan-activated rabbit serum.

[2] Wiggins, R. C., and C. G. Cochrane. The importance of surface for the expression of Hageman factor activity. α-HFa-mediated proteolytic cleavage of Factor XI and prekallikrein. Manuscript submitted for publication.

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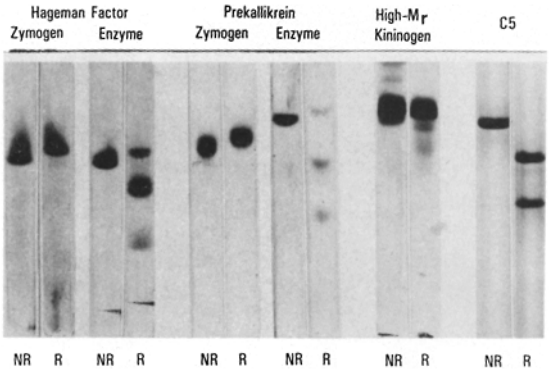

FIG. 1. Proteins purified from rabbit plasma (used in these studies) analyzed by SDS-PAGE in the presence (R) and absence (NR) of β-mercaptoethanol. Zymogen HF is a single polypeptide chain of 82,000 M_r. HFa (enzyme) is dissociated into fragments of 52,000 and 28,000 M_r in the presence of reducing agents. Residual native HF remains at 82,000 M_r in the presence of reducing agents. PK zymogen is a single polypeptide chain of 85,000 M_r. Upon activation to kallikrein, the single polypeptide chain is cleaved into fragments of 55,000 and 35,000 M_r. High-M_r kininogen is a single polypeptide chain of 100,000 M_r in the presence and absence of reducing agents. Rabbit C5 is a two-chain disulfide-linked molecule of unreduced 180,000 M_r, which dissociates into chains of 130,000 and 85,000 M_r in the presence of reducing agent. HF, PK and high-M_r kininogen are shown here on 7.5% SDS gels, whereas C5 is shown as a 5% SDS gel.

goat-anti-rabbit PK was prepared by: (*a*) absorption of plasma with kaolin (25 mg kaolin/ml) incubated for 10 min at 37°C and then centrifuged for 15 min at 5,000 *g* to remove the kaolin; (*b*) dialysis of the kaolin-absorbed serum against 0.01 M phosphate buffer, pH 7.7, followed by absorption in batches using DEAE-cellulose (10 g/g protein) equilibrated in the above buffer; (*c*) concentration of the globulin fraction by precipitation with 50% ammonium sulfate and subsequent dialysis against 0.1 M phosphate buffer, pH 7.5. The IgG fraction thus produced contained no detectable goat kallikrein (<20 ng/ml), as measured by the chromogenic tripeptide assay.

This IgG fraction in 0.1 M phosphate buffer, pH 7.5, was coupled to cyanogen bromide-activated Sepharose-4B (5) at a ratio of 5 mg of protein/1 g of Sepharose-4B. The washed beads were incubated overnight at +4°C with 5 mM diisopropyl fluorophosphate in water. The beads were rewashed and poured into a 2.5- × 30-cm plastic column to a bed height of 26 cm. The column was washed with 5 M guanidine, followed by 0.1 M Tris buffer, pH 7.4, containing 1 M NaCl, 20 mM EDTA, 10 mM benzamidine, 0.05% hexadimethrene bromide (Polybrene; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 0.02% sodium azide.

Citrated normal rabbit plasma (200 ml) was made 10 mM with benzamidine, 0.7 M with NaCl, 20 mM EDTA, 0.05% Polybrene, and 0.02% sodium azide. The plasma was run through the column at room temperature at a flow rate of 100 ml/h. The column was washed with 10-column volumes of starting buffer and then eluted with 5 M guanidine (Sigma Chemical Co., St. Louis, Mo.). The fractions calculated to contain the initial guanidine step and one-column volume thereafter were pooled for the second step.

Step 2. Ion-Exchange Chromatography on SP-Sephadex C-50. The pool from Step 1 (200 ml) was dialyzed against 0.1 M acetate buffer, pH 5.3, containing 0.04 M NaCl, 2 mM EDTA, 1 mM benzamidine, 0.05% Polybrene, and 0.02% sodium azide. 2 g of SP-Sephadex C50 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) hydrated overnight in the above buffer was poured into a siliconized 2.5- × 30-cm column. After two changes of dialysis, the pool was loaded at 40 ml/h. The column was washed with four-column volumes of starting buffer. A step elution was effected with 0.1 M acetate buffer, pH 5.3, containing 0.35 M NaCl (= 40 mS).

Step 3. Affinity Chromatography on Concanavalin A-Sepharose. The pooled fractions containing PK (40 ml) were dialyzed against 0.1 M sodium phosphate buffer, pH 7.5, containing 1 M NaCl, 0.02% sodium azide, and 1 mM benzamidine. The dialyzed material was loaded at 3

ml/h onto a 1.7-cm diameter siliconized glass column containing 6 ml of concanavalin-A Sepharose. The column was washed with 60 ml of the above buffer. Elution of PK was effected with the above buffer containing 0.5 M α-D-methyl glucoside at 2 ml/h. Analysis of the eluted protein from the above column by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and nonreducing conditions showed a single protein band with a molecular weight (M_r) of 83,000 (Fig. 1). Assays of PK were performed by immunodiffusion, using goat anti-rabbit PK antiserum.

KALLIKREIN GENERATION. Kallikrein was generated by incubating PK with α-HFa in the following manner. PK (1 ml, 220 μg) was incubated with α-HFa (50 μl, 10 μg) in the presence of 10 μl 2 M Tris buffer, pH 8.0, for 60 min at 37°C. The HFa was not removed from the kallikrein preparation, but constituted <1% of the protein present. The extent of proteolytic cleavage of prekallikrein as measured by SDS-PAGE (Fig. 1) varied between 58 and 94% in different preparations. The enzymatic activity of kallikrein in each preparation was measured colorimetrically using the tripeptide substrate *N*-Benzoyl-L-Pro-L-phe-L-arginine-*p*-nitroanalide (Bz-pro-phe-arg-pNA; Vega Biochemicals, Tucson, Ariz.) under the conditions described below. The calculated K_{cat} value obtained for different preparations varied between 300 and 400 min^{-1}. The PK purified in this manner showed a line of identity with rabbit PK purified by ion-exchange chromatography (6) and generously donated by Dr. Richard Ulevitch of the Research Institute of Scripps Clinic.

HIGH-M_r KININOGEN. High-M_r kininogen was purified from rabbit plasma by a three-step procedure.

Step 1. Solid-Phase Immunoabsorption of High-M_r Kininogen from Rabbit Plasma. Goat anti-rabbit high-M_r kininogen was coupled to Sepharose 4B as described for PK. Citrated normal rabbit plasma was passed over an anti-high-M_r kininogen-Sepharose 4B column and eluted with 5 M guanidine as described above.

Step 2. DEAE-Sephadex A50 Ion-Exchange Chromatography. The pool from Step 1 (500 ml) was dialyzed against buffer containing 0.4 M Tris, 0.1 M succinic acid, pH 8.2, containing 40 mM NaCl, 10 mM benzamidine, 2 mM EDTA, and 0.02% sodium azide. This material was loaded onto a 2.5- × 30-cm siliconized column containing 3 g of DEAE-Sephadex A50 equilibrated in the starting buffer. The column was washed with five-column volumes of starting buffer and eluted with a gradient consisting of 150 ml of the starting buffer in the proximal chamber and 150 ml of 0.3 M Tris, 0.12 M succinic acid, pH 7.4, containing 0.3 M NaCl, 2 mM EDTA, 10 mM benzamidine, and 0.02% sodium azide. The high-M_r kininogen eluted in the distal shoulder of the protein peak, whereas low-M_r kininogen eluted in the proximal shoulder of the peak.

Step 3. SP-Sephadex Ion-Exchange Chromatography. The pool containing high-M_r kininogen from Step 2 (70 ml) was dialyzed against 0.1 M acetate buffer, pH 5.3, containing 75 mM NaCl, 10 mM benzamidine, 2 mM EDTA, and 0.02% sodium azide. This material was loaded onto 2 g SP-Sephadex C50 equilibrated in the above buffer. The column was washed with 10-column volumes of the above buffer with the NaCl increased to 60 mM, and the high-M_r kininogen was then eluted with a salt gradient to 0.4 M NaCl in 0.1 M acetate buffer, pH 5.3. The eluted material analyzed by SDS-PAGE in the presence and absence of reducing agents revealed a single predominant protein band with an M_r of 100,000 (Fig. 1). This material had a specific clotting activity of 6.5 clotting U/mg, where 1 U is the amount of rabbit high-M_r kininogen present in 1 ml of plasma when assayed in human high-M_r kininogen-deficient (Fitzgerald) plasma.

C5. C5 was purified from normal rabbit plasma by euglobulin precipitation (pH 5.0), and ion-exchange and gel-filtration chromatography as previously described (7). Rabbit C5 thus prepared was homogeneous on SDS-PAGE (Fig. 1) and contained no detectable C3 by functional or immunochemical analysis.

EAC423 Preparation. Sheep erythrocytes bearing the classical pathway C5 convertase were prepared from human complement components by the method of Rapp and Borsos (8).

Chromogenic Tripeptide Assay. The synthetic chromogenic tripeptide Bz-pro-phe-arg-pNA HCl was dissolved in 0.1 M Tris, pH 8.3, containing 0.05 NaCl and 0.02% sodium azide at a final concentration of 0.1 mg/ml. The sample to be assayed (2–5 μl) was added to a 300-μl volume of substrate in a cuvette and the change in absorbance was measured at 405 nM. The

values for K_{cat} were calculated using a molar extinction coefficient of 10,000 M^{-1} cm^{-1} at 405 nM.

Neutrophil Function Assays. These were performed using leukocytes isolated from New Zealand White rabbits as previously described (9). The modified Boyden assay was performed using the two-filter technique of Keller et al. (10). The leading front method was performed according to Zigmond and Hirsch (11). Each sample was measured in triplicate and the results were expressed as the mean ± 1 SD from the mean. For each assay positive (5% zymosan-activated rabbit serum [ZARS] and negative (Hanks' balanced salt solution-bovine serum albumin [HBSS-BSA]) controls were incorporated. The Keller assay and leading front assays were sensitive to 5 ng and 0.2 ng of human C5a, respectively (9).

Kinin Bioassay. This was performed using the estrogen-sensitized rat uterus assay as previously described (12).

SDS-PAGE. Analysis by SDS-PAGE was performed using cylindrical gels and the Weber-Osborne system (13). Gels were stained for protein with Coomassie Blue dye or cut into 1.2-mm slices and counted for ^{125}I on an automated γ-counter (Searle Radiographics, Inc., Des Plaines, Ill.).

Miscellaneous. Protein determinations were performed by the method of Lowry et al. (14) using BSA (5 times recrystallized; Reheis Chemical Co., Chicago, Ill.) as a standard. Radiolabeling was performed with ^{125}I using the chloramine T method (15). Neutrophil enzyme release was measured as described in reference 16.

Results

Chemotactic Activity Generated from C5 by Plasma Kallikrein. When rabbit C5 was incubated with rabbit plasma kallikrein, chemotactic activity could easily be demonstrated (Table I). In contrast, no chemotactic activity could be demonstrated when the zymogen PK was tested with C5 under identical conditions. Chemotactic activity was also generated when rabbit C5 was incubated with α-HFa, trypsin, (Table I), or EAC423. No chemotactic activity was produced when C5 was absent from the incubation mixtures, or when intact C5 alone was assayed.

Direct Chemotactic Effect of Plasma Kallikrein. Because plasma kallikrein has been reported to be directly chemotactic for neutrophils (4), we investigated the ability of rabbit plasma kallikrein alone to attract rabbit neutrophils. As shown in Table II, over a dose-range from 2.5×10^{-7}–10^{-10} M kallikrein, no detectable chemotactic

TABLE I

Reagent	Chemotactic activity (cells/HPF* ± 1 SD)	
	No C5	Added C5
Buffer	2.4 ± 2.2	2.0 ± 0.9
Kallikrein	0.6 ± 0.4	50.1 ± 3.8
PK	0.2 ± 0.3	1.8 ± 0.4
α-HFa	1.5 ± 2.3	25.8 ± 7.7
Trypsin	1.5 ± 1.2	20.4 ± 1.5
EAC423	3.0 ± 0.9	18.2 ± 2.4
ZARS	87.8 ± 23.6	—

Chemotactic activity was generated when kallikrein (1.4 μg), α-HFa (2.4 μg), trypsin (0.2 μg), and EAC423 cells (1×10^7) were incubated with C5 (10 μg). All incubations were for 20 min at 37°C with the exception of trypsin, which was for 2 min at 22°C. The reaction was stopped with SBTI (40 μg) added for 5 min at 22°C. ZARS (5%) was used as a positive control. Each assay was performed in triplicate.

* High power field.

TABLE II
Effect of Plasma Kallikrein upon Neutrophil Chemotaxis

Plasma kallikrein	Boyden chamber (cells/HPF* ± 1 SD)	Leading front (distance ± 1 SD)
M		
2.5×10^{-7}	1.6 ± 1.2	14.3 ± 0.6
10^{-7}	3.2 ± 2.0	
2.5×10^{-8}	0.7 ± 0.1	23.0 ± 4.6
10^{-8}	0.6 ± 0.2	
2.5×10^{-9}	1.5 ± 1.6	26.7 ± 3.8
10^{-9}	0.6 ± 0.6	
2.5×10^{-10}		27.7 ± 4.9
Buffer control	0.8 ± 0.4	29.3 ± 5.1
5% ZARS	87.8 ± 4.0	145.0 ± 20

Plasma kallikrein at the above concentrations was tested for chemotactic activity in the presence of HBSS containing BSA (20 mg/ml) by both the modified Boyden assay and the leading front method (n = 3).
* High power field.

activity was observed using the modified Boyden chamber (see Materials and Methods). This was found consistently in four experiments using two different kallikrein preparations. Furthermore, using the more sensitive leading front method (see Materials and Methods), again, no chemotactic activity could be detected (Table II) in two experiments.

Enzymatic Activity of the Rabbit Plasma Kallikrein

To confirm that the kallikrein used in these experiments was biologically active, the following assays were performed.

TRIPEPTIDE HYDROLYSIS. The rate of hydrolysis of the synthetic substrate Bz-pro-phe-arg-pNA was measured spectrophotometrically. The calculated K_{cat} value ranged from 300 to 460 min^{-1} under the conditions of assay (see Materials and Methods).

PROTEOLYTIC CLEAVAGE OF RADIOLABELED SUBSTRATES. The ability of the kallikrein to cleave its natural substrates, HF and high-M_r kininogen, was measured. ^{125}I-HF (200 μg, 0.2 μCi) or ^{125}I high-M_r kininogen (250 μg, 0.2 μCi) were incubated with dilutions of kallikrein and PK in the presence of 0.1 M Tris, pH 7.4, and BSA (1 mg/ml). Typical cleavage patterns are shown in Fig. 2, where ^{125}I-HF was cleaved into fragments of 28,000 and 50,000 M_r, and ^{125}I high-M_r kininogen was cleaved to one major fragment containing ^{125}I with an M_r of 70,000.

KININ GENERATION. The ability of kallikrein to release biologically active kinin from rabbit high-M_r-kininogen was measured using an estrogen-sensitized rat uterus bioassay system (Table III).

As confirmed by these studies, the kallikrein was capable of releasing biologically active kinin from high-M_r kininogen in association with proteolytic cleavage of the molecule. In contrast, PK did not cleave high-M_r kininogen and did not generate kinin. However, if α-HFa was incubated with PK, the PK was activated with subsequent release of kinin from high-M_r kininogen. We conclude that all the proteins used in these studies were capable of biological activity and that the kallikrein preparation was indeed kallikrein.

Chemotactic Effect of HF and High-M_r Kininogen. Because kallikrein appeared not to

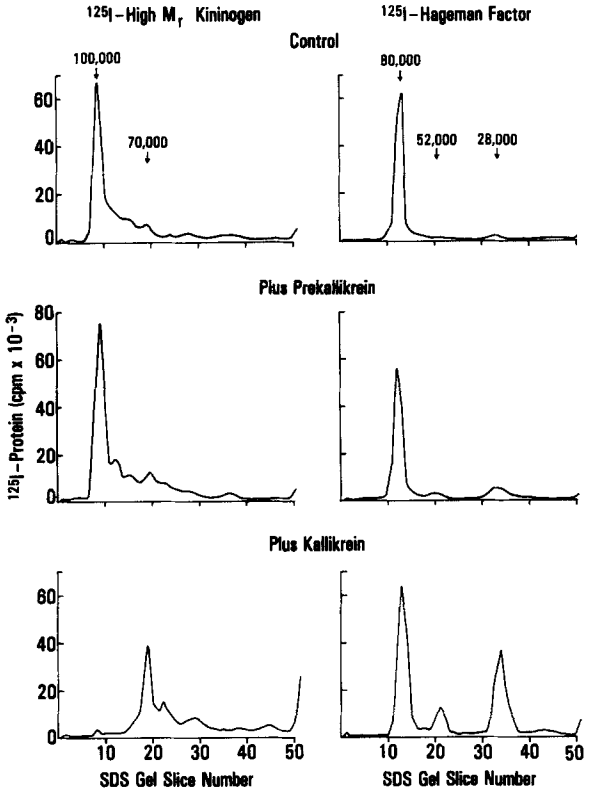

FIG. 2. Radioactivity profiles from reduced SDS-PAGE of ^{125}I-HF and ^{125}I-high-M_r kininogen incubated with PK or with kallikrein. Kallikrein caused proteolytic cleavage of high-M_r kininogen into a major ^{125}I-containing fragment of 70,000 M_r. Kallikrein caused cleavage of ^{125}I-HF into fragments of 52,000 and 28,000 M_r.

TABLE III

Kinin Generation by Purified Components of the Rabbit HF System

Kallikrein	PK	α-HFa	High-M_r kininogen	Response time	Bradykinin equivalents
0.9 μg	*0.9 μg*	*1.8 μg*	*7 μg*	*s*	*ng*
+	−	−	+	3.0	38.0
−	+	−	+	NR	<4
−	−	+	+	NR	<4
−	+	+	+	3.5	30.4
+	−	−	−	NR	<4
−	+	+	−	NR	<4

Purified proteins of the rabbit HF system were incubated together for 10 min at 37°C in the presence of 0.05 M Tris buffer containing BSA (1 mg/ml) in a total volume of 80 μl. 100 μl of hot (100°C) saline was added to each tube and the samples were boiled for 4 min before the assay using an isolated estrogen-sensitized rat uterus (see Materials and Methods). NR, no response.

have any chemotactic activity, the natural kallikrein substrates in their activated form (α-HFa and kinin-free high-M_r kininogen) were tested for their ability to attract cells.

α-HFa, the two-chain, 80,000-M_r form of activated HF, was tested over a dose

range of 7×10^{-7}–7×10^{-10} M using the Boyden assay system. No chemotactic activity was observed (data not shown).

Rabbit high-M_r kininogen (220 μg) was preincubated with rabbit kallikrein (20 μg) for 30 min at 37°C in the presence of 0.1 M Tris, pH 7.4. No chemotactic activity could be detected in this incubation mixture over a dose range of 2.5×10^{-7}–2.5×10^{-10} M high-M_r kininogen (data not shown).

We conclude that neither kallikrein nor HFa nor high-M_r kininogen is capable of attracting rabbit neutrophils under the conditions of study.

Finally, PK (40 μg/ml), HF (22 μg/ml), α-HFa (3 μg/ml), and high-M_r kininogen (55 μg/ml) were incubated together for 20 min at 37°C in the presence of BSA (1 mg/ml). Activation of protein was confirmed by measurement of kinin generation by bioassay. No chemotactic activity could be demonstrated using the Boyden method. We conclude that no detectable chemotactic fragments were generated during activation of these proteins.

Dose-Response Curve of Generation of Chemotactic Activity from C5 by Kallikrein. A dose-response curve of rabbit plasma kallikrein incubated with rabbit C5 (at a final concentration of 100 μg/ml) demonstrated the production of chemotactic activity by kallikrein down to a level of ~1 μg/ml (Fig. 3) (the physiological concentration of PK in rabbit plasmas is ~25 μg/ml). Thus, plasma kallikrein at physiological concentration appears to be capable of generating chemotactic activity from C5.

As indicated in Table I, α-HFa also generated chemotactic activity from C5. A dose-response curve for α-HFa indicated that α-HFa was about one-third as active as kallikrein in this respect.

Kallikrein-blocking Studies. To establish whether the kallikrein effect on C5 could be due to a contaminating enzyme, blocking studies were performed with anti-kallikrein IgG and with soybean trypsin inhibitor (SBTI). Rabbit kallikrein (20 μg/ml) was incubated with anti-PK or anti-HF antisera at a final concentration of 8 mg/ml for 20 min at 22°C in the presence of 0.1 M Tris buffer, pH 7.4. The kallikrein preparations were then assessed for their ability to (*a*) hydrolyse Bz-pro-phe-arg-pNA; (*b*) generate kinin from purified high-M_r kininogen as measured by the rat uterus bioassay; and (c) generate chemotactic activity from C5. For each assay system, the amount of the kallikrein-anti-HF preparation (control) used was on the upper portion of the dose-response curve. The response of the kallikrein-anti-PK preparation was

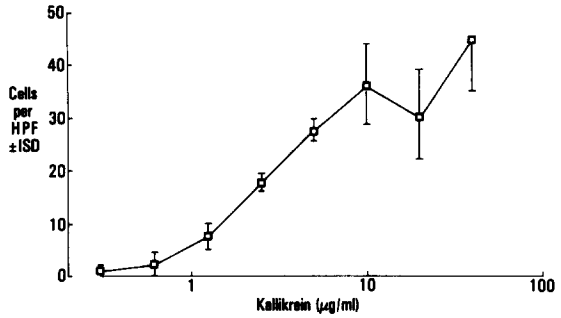

FIG. 3. Dose-response curve for kallikrein-induced chemotaxis in the presence of C5 (10 μg). The final incubation volume was 100 μl in the presence of 0.05 M Tris, pH 7.6, containing 0.5 mg/ml ovalbumin. After incubation for 20 min at 37°C, SBTI (40 μg) was added for 5 min at 22°C before chemotaxis assay.

compared with this value and expressed as the percent of the control. As shown in Fig. 4, the anti-PK IgG blocked the tripeptide hydrolysis response by only 26%, whereas the chemotaxis and kinin generation responses were blocked by 41 and 68%, respectively. This difference in blocking effect by antibody was reproducible and was not effected by changing the ratio of kallikrein to anti-PK. It probably reflects greater steric hindrance by the anti-PK IgG-kallikrein complex for the larger substrates and possibly the requirement for two clips to liberate kinin from kininogen.

Kallikrein (5 μg) was also incubated with various doses of STBI (2.9, 1.5, and 0.7 μg) to generate inactive SBTI-kallikrein complexes in varying proportions such that ~¼, ½, and ¾ of the kallikrein molecules would remain active. These partially inhibited kallikrein preparations were compared with a noninhibited control for their ability to (*a*) hydrolyse the tripeptide Bz-pro-phe-arg-pNA; (*b*) generate bradykinin from purified high-M_r kininogen; and (*c*) generate a chemotactic response from C5. As shown in Fig. 5, the ability of SBTI to inhibit kallikrein activity on three different substrates was similar, indicating that the kallikrein-induced chemotactic response was due to kallikrein itself and not due to a contaminating enzyme.

Nature of the Chemotactic Substance Generated from C5 by Kallikrein

SECRETION OF β-GLUCURONIDASE FROM NEUTROPHILS. C5a is capable of causing specific release of granular contents from cytochalasin B-treated neutrophils (9). To determine whether kallikrein-treated C5 generated a fragment capable of causing specific release as well as chemotaxis, the following experiment was performed.

Isolated rabbit neutrophils were suspended in HBSS in the presence of BSA (0.25%).

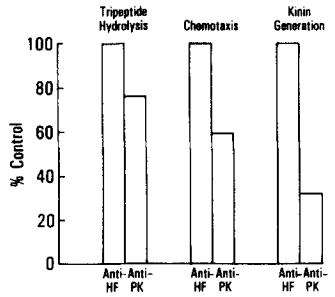

FIG. 4. Comparison of the effect of anti-PK IgG and anti-HF IgG upon the ability of kallikrein to (*a*) hydrolyze the tripeptide Bz-pro-phe-arg-pNA; (*b*) generate chemotactic activity from C5; and (*c*) liberate kinin from high-M_r kininogen.

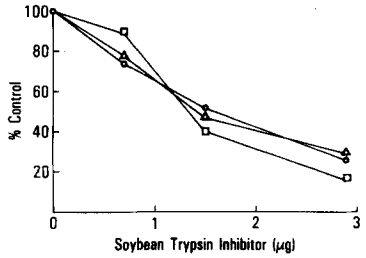

FIG. 5. Inhibitory effect of SBTI upon the ability of kallikrein to (*a*) hydrolyze the tripeptide Bz-pro-phe-arg-pNA (○); (*b*) generate chemotactic activity from C5 (△); and (*c*) liberate kinin from high-M_r kininogen (□).

Aliquots containing 4×10^6 neutrophils were incubated for 5 min at 37°C with cytochalasin B (5 μg/ml prepared at 5 mg/ml in dimethyl sulfoxide). The cells were then exposed to C5 (25 μg), which had been preincubated for 120 min at 37°C either with buffer, kallikrein (550 ng), EAC423 (5×10^7 cells), or trypsin (300 ng), after which SBTI (1 μg) was added to each incubation mixture and allowed to stand for 5 min at 22°C. Purified human C5a (15 ng) (generously donated by Robert O. Webster, National Jewish Hospital and Research Center, Denver, Colo.) was used as a positive control. The release of a granular enzyme, β-glucuronidase, and a cytosol enzyme, lactic dehydrogenase (LDH), was measured as previously described (16, 17) after 30 min incubation with the stimulus. No significant release of LDH occurred (<5% of total releasable by detergent), indicating that no cell lysis had occurred. The base-line release of β-glucuronidase with C5 alone after 120 min at 37°C was 9.8% of the total enzyme releasable by detergent lysis from an equivalent number of cells. In contrast, incubation of C5 with kallikrein, EAC423 cells or trypsin resulted in 45.3, 51.4, and 49.4% β-glucuronidase release, respectively. Purified human C5a caused 53.4% release. Thus kallikrein, like EAC423 cells and trypsin, generated from C5 a factor that caused rabbit neutrophils to release a specific granular enzyme.

EFFECT OF CARBOXYPEPTIDASE N. Carboxypeptidase N cleaves the C-terminal arginine from human C5a to generate C5a des arg, a molecular species with 100-fold less secretogogue activity than C5a (9). To determine whether the chemotactic factor generated from C5 by plasma kallikrein was susceptible to carboxypeptidase N, the following experiment was performed. Kallikrein or trypsin was incubated with C5 under the conditions described above. Human C5a and the rabbit C5-kallikrein and C5-trypsin incubates were treated for 5 min at 22°C with 1 mg SBTI to inhibit the enzymes and then incubated with or without carboxypeptidase N (endopeptidase-free; Worthington Biochemical Corp., Freehold, N. J.), 1% carboxypeptidase N (wt/wt) in 1% $NaHCO_3$, pH 7.0, for 15 min at 37°C. The ability of human C5a and the C5-kallikrein and C5-trypsin incubates to release β-glucuronidase in the absence of carboxypeptidase N were 64, 61, and 60% of the total detergent-releasable enzyme, respectively. After incubation with carboxypeptidase N, the respective values were 18, 25, and 16%. Thus, carboxypeptidase N partially inhibited the specific release of β-glucuronidase from neutrophils by the kallikrein C5 incubate. Furthermore, the extent of inhibition was similar to that seen for carboxypeptidase-treated human C5a.

CLEAVAGE FRAGMENTS OF C5 GENERATED BY KALLIKREIN. Studies were performed to determine the size of cleavage fragments generated from C5 by incubation with kallikrein. Fragments of C5 were visualized by both Coomassie Blue staining and autoradiography of ^{125}I-C5 before and after incubation with kallikrein. Analysis of the cleaved proteins in the presence and absence of reducing agents was performed on exponential gradient (8–20%) acrylamide gels in the presence of SDS. In the absence of reducing agents, a single cleavage fragment was consistently seen by autoradiography with an apparent molecular weight of ~14,000. In the presence of reducing agents, only the α-chain of rabbit C5 appeared to be cleaved when analysed both by autoradiography and stained gels. In the presence of reducing agents, fragments were observed with apparent molecular weights of 50,000, 34,000, and 25,000 in addition to the 14,000-M_r fragment.

These findings indicate that plasma kallikrein can release a 14,000-M_r fragment

from C5 and that the C5 molecule may also be cleaved by kallikrein at additional sites. The 14,000-M_r fragment could be a chemotactic C5a-like molecule.

Generation of Chemotactic Activity in Kaolin-activated Plasma. Addition of kaolin to plasma results in activation of the HF system with consequent kallikrein activation. Previous studies have reported both that chemotactic activity is (4) or is not (18) generated during kaolin activation of plasma. To resolve this discrepancy, rabbit plasma (containing 0.38% sodium citrate) was activated with kaolin (10 mg/ml final concentration, 10-min incubation at 37°C) under conditions that activate >70% of PK to kallikrein. As a negative control, citrated plasma was used, and as a positive control, citrated plasma was activated with zymosan. Each plasma was assayed for chemotactic activity in triplicate. The results are shown in Table IV. Although there was a consistent trend for kaolin-activated plasma to contain more chemotactic activity than control plasma, this was only statistically significant when low concentrations of plasma were used. These results and other studies consistently indicated that kaolin-activated plasma tends to contain more chemotactic activity than control plasma but much less chemotactic activity than zymosan-activated plasma. However, when kaolin was added to zymosan-activated rabbit plasma at a final concentration of 50 mg/ml, the chemotactic activity was reduced from 58.7 ± 10.3 to 3.5 ± 1.6 ($n = 3$, $P < 0.01$). Thus, at high concentrations kaolin blocks detectable chemotactic activity, probably by binding positively charged C5a to the negatively charged kaolin surface.

Therefore, although the assessment of chemotactic activity in kaolin-activated plasma is complicated by the probable binding of C5a to kaolin, the chemotactic activity of plasma did appear to increase after incubation with kaolin.

Discussion

Previous studies using partially purified preparations of human plasma kallikrein have indicated that kallikrein itself might be directly chemotactic for neutrophils (4, 19–21). This observation is surprising, because no other enzyme is known to have direct chemotactic activity. In particular, trypsin, with its ability to hydrolyse peptide

TABLE IV

*Chemotactic Activity in Cells/HPF**

Plasma concentration used	Control plasma, (mean ± 1 SD)	Kaolin-activated plasma, (mean ± 1 SD)	Zymosan-activated plasma, (mean ± 1 SD)
%			
0.5	3.2 1.8	5.5 0.1‡	71.3 38.2‡
1.0	3.1 1.5	4.9 0.8	77.4 20.9§
5.0	4.5 2.4	8.4 2.2	107.6 25.9§
10.0	5.0 1.6	5.6 1.7	103.1 21.6§

Chemotactic activity present in plasmas after incubation alone or with kaolin (5 mg/ml) or zymosan (5 mg/ml) for 10 min at 37°C. Each sample was tested in triplicate as described in Materials and Methods. Section marks indicate indicate the degree of significance between the treated and control plasmas as indicated by a paired *t* test.

* High power field.

‡ $P < 0.05$.

§ $P < 0.01$.

bonds adjacent to positively charged residues in a rather unrestricted fashion, is not itself chemotactic. This contrasts with the fact that the trypsinlike arginine esterase plasma kallikrein appeared to require enzymatic activity for its chemotactic activity (4).

The findings from this study, using highly purified and well characterized proteins and cells of rabbit origin, indicate that rabbit plasma kallikrein is not chemotactic. In addition, HF, HFa, high-M_r kininogen, activated high-M_r kininogen, and PK were not chemotactic alone or when incubated together so that activation occurred in the incubation mixture. Thus, activation of the purified proteins of the HF system did not generate a chemotactic fragment that was detectable under the conditions employed in this study.

However, plasma kallikrein was capable of producing a chemotactic effect if C5 was included in the incubation mixture. Plasma kallikrein at concentrations as low as 1 μg/ml appeared to be capable of generating a chemotactic effect from C5 when the latter was present at physiological concentrations (100 μg/ml). It is unlikely that this effect was due to a contaminating protease that was not detected in the kallikrein preparation by SDS or alkaline gel analysis, because specific anti-kallikrein IgG inhibited kallikrein-mediated kinin generation and kallikrein-mediated chemotaxis to a similar extent. The finding that the antibody only partially inhibited both effects provides stronger evidence that the same enzyme was involved. Furthermore, SBTI also inhibited the kinin-generating, chemotactic, and tripeptide-hydrolysing effects to the same extent. We conclude that the chemotaxis generation was due to the action of plasma kallikrein on C5.

The nature of the chemotactic substance generated from C5 by kallikrein appeared to be similar to C5a. Thus, it was chemotactic, it stimulated β-glucuronidase release from cytochalasin B-treated neutrophils, it was sensitive to carboxypeptidase N digestion, and its activity was associated with the generation of a low-M_r fragment (~14,000) from C5. In addition, the amount of chemotactic and secretagogue activity generated from C5 by kallikrein was consistent with calculations of the amount of C5a activity that could be released from the quantity of C5 in the initial incubation mixture. Furthermore, kallikrein, like trypsin, is an arginine esterase and the C-terminal amino acid of both human and porcine C5a is arginine (22). From the above considerations, it seems likely that C5a can be released from rabbit C5 by plasma kallikrein.

The potential importance of kallikrein in liberating C5a from C5 in vivo is not yet known. However, during tissue injury, substances such as bacterial lipopolysaccharides (23), collagen and elastin (24–26), basement membrane (27), and sodium urate and pyrophosphate crystals (28) may act as negatively charged surfaces upon which assembly and activation of the molecules of the HF system can occur (29). Damaged endothelial cells contain an enzyme capable of directly activating HF (12). Under these conditions, kallikrein could be generated that might then release C5a from C5. The importance of the capacity of C5a to bind to negatively charged substances, thereby creating a stable surface-bound gradient for orientation of migrating neutrophils, has previously been emphasized (30). Thus, it is not surprising that negatively charged agents, such as kaolin, used to activate the HF system in the laboratory, should also have the capacity to bind C5a. Although this fact makes the measurement of C5a in the presence of a kallikrein-generating system in plasma more difficult, it

may reflect the importance of exposure of negatively charged substances such as basement membrane, collagen, or elastin during tissue injury. It is possible that these negatively charged substances may not only allow activation of the HF system with kallikrein generation, but may also bind C5a locally generated by kallikrein with consequent effects on vascular permeability and attraction of two major effector cells of inflammation: neutrophils and macrophages (31–34).

There remains the apparent discrepancy between the findings described in this study using rabbit proteins and previous observations using human proteins (4, 19–21). One possible explanation for this discrepancy would be that human leukocytes have a membrane-associated C5-9 complex (35). Human kallikrein might therefore release C5a from membrane-bound C5 and thus appear to be chemotactic in the assay system. It is possible that the concentrations of rabbit kallikrein used in this study were not sufficient to observe this effect with rabbit leukocytes.

Summary

Rabbit plasma kallikrein incubated with rabbit C5 resulted in the generation of chemotactic and secretagogue activity for rabbit neutrophils. This effect on C5 appeared to be due to kallikrein itself and not to a contaminating enzyme, because it could be inhibited by anti-kallikrein IgG or by soybean trypsin inhibitor to the same extent that kinin generation by the same kallikrein preparation was inhibited by these agents. The chemotactic response was consistent with the generation of a C5a-like peptide from C5 because the effect could be partially inhibited by carboxypeptidase N and was related to the generation of a small (~14,000 mol wt) fragment of C5.

No direct chemotactic response was detectable for kallikrein, activated Hageman factor, high-molecular weight kininogen, or intact C5. Incubation of kallikrein, high-molecular weight kininogen, and Hageman factor together, so that activation of all three proteins occurred, did not result in the generation of detectable chemotactic activity.

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