A POTENT vasoactive peptide, bradykinin, is liberated in plasma from two precursor proteins, high- and low-mol wt kininogens (HMWK and LMWK), by the action of specific enzymes termed kallikreins.\(^1\)\(^2\) Human HMWK and LMWK are single-chain glycoproteins, with mol wts of ~110,000 to 120,000 and 50,000 to 70,000, respectively. Each protein consists of three domains: an amino terminal heavy chain, a bradykinin moiety, and a carboxyl terminal light chain.\(^3\)\(^4\) Based on cloning and sequence analysis of cDNAs for human high- and low-mol wt prekininogens, a complete identity in the amino acid sequence has been determined for a large portion of the two molecules, including the heavy chain, the bradykinin sequence, and the first part of the carboxyl terminal region; the two proteins are transcribed from the same gene.\(^5\) The structural homology accounts for the recently discovered thiol protease inhibitory activity common to both proteins.\(^6\)\(^7\)

The unique structure of the light chain of HMWK, however, provides this protein with the function of accelerating the reactions of the contact phase of blood coagulation, whereby HMWK acts as a nonenzymatic cofactor by facilitating the proteolytic activations of factor XII, prekallikrein, and factor XI on a negatively charged surface.\(^9\)\(^10\) One of the activation products, kallikrein, potently cleaves HMWK at two specific sites to liberate bradykinin and to form a kinin-free protein.\(^11\)\(^12\) The cleavage occurs within a critical disulfide bridge so that single-chain HMWK is converted to a two-chain molecule, in which a heavy chain of ~66,000 mol wt is disulfide-linked to a light chain of ~55,000 mol wt. The light chain is further cleaved by kallikrein to a smaller chain of 45,000 mol wt.\(^13\)

The pathophysiologic role of plasma HMWK still remains largely unclear. The hereditary deficiency of this protein is not associated with a bleeding disorder.\(^14\) Because bradykinin possesses a number of actions potentially involved in inflammation reactions, including vasodilation, increase in vascular permeability, and pain generation, a role for HMWK and the other proteins of the contact activation system in the pathogenesis of several pathological conditions has been suggested.\(^14\) Evidence of HMWK cleavage in vivo, however, has been indirect only and has involved functional and/or immunological measurements of plasma levels of the protein or its released product, bradykinin.

We have prepared a series of monoclonal antibodies (Mabs) against HMWK and describe here their use as specific qualitative and quantitative probes for HMWK in plasma samples subjected to immunoblotting.

### MATERIALS AND METHODS

#### Materials

The following reagents were obtained from sources as indicated: rabbit antibodies against the Fc region of mouse immunoglobulins (Cappel Lab, Westchester, Pa); rabbit antibodies against subtypes of mouse heavy and light chains (Miles Lab, Eckhardt, Ind); Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% newborn calf serum, 10% NCTC (National Cancer Tissue Culture) 109 medium, pyruvate (Microbiological Associates, Bethesda, Md).
Hepes, glutamine, penicillin, streptomycin (GIBCO, Grand Island, NY); diithiothreitol (DTT, Boeringer Mannheim, FRG); 0.45 μm nitrocellulose plastic tubes (Bio Rad, Richmond, Calif); Na-
(Amersham International, England); diethyaminoethanol (DEAE)-Sephadex A50, SP-Sephadex C-50, Protein A-Sepharose CL-4B, mol wt standards (Pharmacia Fine Chemicals Inc, Piscataway, NJ); all other reagents were the best grade available.

HMWK-deficient plasma was purchased from George King Bio-Medical Inc, Overland Park, Kan. Plasma samples from a patient with hereditary Cl-inhibitor deficiency were collected in 3.8% sodium-citrate using plastic tubes during and after an attack of angioedema that did not require any specific therapy. A normal human plasma (NHP) pool or individual plasma samples were prepared in our laboratory by collecting blood from 17 normal male subjects according to standard procedures. Informed consent was obtained from normal subjects and patients. Blood was usually anticoagulated with 3.8% sodium-citrate (9:1/vol:vol) and, in some experiments, blood contained benzamidine (8 mmol/L) or soybean trypsin inhibitor (SBTI, 100 μg/mL) or leupeptin (0.25 mmol/L). Plasma from EDTA (10 mmol/L) or heparin (10 IU/mL) anticoagulated blood, with or without inhibitors, was used where indicated.

**General Methods**

Sodium dodecyl sulfate (0.1%) polyacrylamide gel electrophoresis (SDS-PAGE) was performed in slab gels (1.5 mm thick) according to the methods of either Weber et al or Laemmli. Samples (purified proteins or plasma) were mixed with appropriate sample buffer containing 2.5% SDS, heated for five minutes at 100 °C, and applied to the gels. After electrophoresis, gels were immediately used for immunoblotting (see below) or stained with Coomassie Brilliant Blue R250. Apparent mol wts were determined by running standard proteins of known mol wt (range 14,000 to 94,000 mol wt) on the gel. In some experiments, unstained duplicate gels were sectioned into 1-mm segments, and each slice was suspended in 0.2 mL of 0.01 mol/L of Tris, pH 7.4 containing 0.14 mol/L of NaCl and 1 mg/mL of bovine serum albumin (TBS-BSA) overnight at 4 °C. After extensive dialysis against TBS-BSA and protein concentration measurements, eluates of selected slices and control eluates were assayed for HMWK clotting activity and used for cleavage experiments by human plasma kallikrein, as described below.

Radioiodination of HMWK was performed by the chloramine T method. 125I-HMWK had a specific radioactivity of 4.5 μCi/μg protein and retained its original clotting activity after the radiola beling procedure. The same method was used to obtain 125I-rabbit anti-mouse IgG with a specific radioactivity of ~1 to 3 μCi/μg.

The procoagulant activity of HMWK was measured by a one-stage assay, using a congenital deficient plasma as a substrate and an automated coagulometer (COAG-A-MATE 2001, General Diagnostics, Morris Plains, NJ) to record the activated partial thromboplastin time.

Protein determination and immunodiffusion were performed according to Lowry et al and Ouchterlony and Nilsson, respectively.

**Protein Purification**

HMWK was purified from human plasma as previously described. Human plasma kallikrein, produced by proteolytic activation of prekallikrein with β-factor Xa, was used to cleave HMWK and to prepare the two-chain, kinin-free molecule, from which the purified and alkylated heavy and light chains were prepared using SP-Sephadex C-50 chromatography.

**Production of Mabs**

Murine Mabs to HMWK were prepared by a modification of the method of Köhler and Milstein. In brief, female BALB/c mice were each injected intraperitoneally with 50 μg of purified HMWK mixed with complete Freund's adjuvant for the first injection (day 1) and thereafter with antigen in incomplete Freund's adjuvant (day 16). Three days prior to cell fusion (day 35), 50 μg of HMWK was injected intravenously (IV). Spleen cells (8.6 × 10^8) from three immunized mice were fused with P3X63Ag8.653 murine myeloma cells (1.7 × 10^6) using 30% (wt/vol) polyethylene glycol-1000. After two days of 4 × 10^7 mol/L aminopterin treatment, the cells were seeded into 96-well microtiter plates at 1.5 × 10^3 cells per well. Fused cells were cultured in DMEM supplemented with 20% newborn calf serum, 10% NCTC 109 medium, oxalacetate (1 mmol/L), pyruvate (0.45 mmol/L), glutamine (2 mmol/L), penicillin and streptomycin, Hepes (20 mmol/L), hypoxanthine (1 × 10^-4 mol/L), and thymidine (3 × 10^-3 mol/L). After 15 days, supernatants were tested for reactivity against purified HMWK by a solid-phase radioimmunoassay (SPRIA). For these assays, each well of 96-well plastic microtiter plates was coated, overnight at 4 °C, with 0.01 mol/L phosphate, pH 7.4, 0.14 mol/L of NaCl (PBS) containing 100 ng of single-chain HMWK, two-chain HMWK, the isolated alkylated light chain, or the isolated alkylated heavy chain. The plates were washed three times with PBS and patted dry, and 200 μL of blocking buffer (3% BSA, 0.02% NaN3 in PBS) was added and incubated for one hour at 37 °C. Plates were again washed three times with PBS; then hybridoma culture supernatants were added in a volume of 50 μL per well and incubated for two hours at 37 °C. Plates were washed three times with PBS; then 50 μL of 125I-labeled rabbit anti-mouse IgG (200,000 cpm per well in blocking buffer) was added to each well and incubated at 37 °C for two hours. Plates were again washed three times with PBS, and the radioactivity of each cut plastic well was counted, using a Microedic 4/600 automatic γ counter. Ten of 193 positive hybridomas were selected for cloning by limiting dilution. Thymus cells (5 × 10^5/mL) from BALB/c mice were added to the wells as a feeder layer during cloning. Of 526 clones that were again found positive for antibodies against HMWK by the SPRIA, 8 were recloned to ensure monoclonality; 212 clones from the second cloning were again screened by SPRIA and six cell lines were selected for injection (1 × 10^6 cells per animal) into the peritoneal cavity of pristane-primed BALB/c mice. The ascitic fluid obtained from each mouse was tested by a SPRIA and by a dot-immunobinding assay. For the latter test, various proteins including purified single-chain or two-chain HMWK, the purified heavy and light chains, or control proteins (Hageman factor, prekallikrein, factor XI, albumin) were directly spotted onto a nitrocellulose membrane. The immunodetection of the antigen was then accomplished by a procedure identical to that described below under "Immunoblotting." Of 36 ascitic fluids, six were further processed to purify the immunoglobulin fractions by Protein A-Sepharose CL-4B affinity chromatography. A purified murine Mab to human protein C that was produced in our laboratory by the same techniques was used as a control in some experiments.

**Effect of Mabs on HMWK Procoagulant Activity**

Fifty microliters of serial dilutions of purified anti-HMWK Mabs (100, 50, 25, and 10 μg/mL), control Mabs (100 μg/mL) or TBS-BSA were incubated with equal volumes of NHP or purified HMWK (70 μg/mL) in 12 × 75 mm polystyrene tubes at 37 °C for one hour. The mixtures were then assayed for HMWK coagulant activity in comparison to a calibration curve made with serial dilutions of NHP, and the results were expressed as the percentage of activity relative to that found in the control.

**Effect of Mabs on the Binding of HMWK to Kaolin**

125I-HMWK (10 μL) was incubated with 50 μL of NHP or purified HMWK (70 μg/mL), and 40 μL of Mabs (100 μg/mL) or...
TBS-BSA, in a polystyrene tube for one hour at 37 °C. Then 100 μL of kaolin suspensions (10, 5, and 1 mg/mL, final concentrations) were added, and the mixtures were incubated at 37 °C for 15 minutes. The tubes were centrifuged for two minutes at 5,000 g, and the kaolin pellet was counted for radioactivity. Binding of 125I-HMWK was expressed as the percentage of the total radioactivity that was found in the pellet.

Immunoblotting

The original method of Towbin et al was modified as follows: after a 7.5% SDS-PAGE in a discontinuous buffer system, proteins were electrotransferred at 5 V/cm for two hours to a nitrocellulose membrane in a TE-S2 Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco) filled with transfer buffer (50 mmol/L of Tris, 45 mmol/L of glycine, 20% vol/vol methanol, 0.02% SDS). After transfer, the nitrocellulose membrane was incubated in a blocking solution termed “BLOTTO” consisting of 5% wt/vol nonfat dry milk, 0.01% antifoam A, 0.0001% merthiolate, 1 μmol/L of p-amidinophenyl-methyl-sulfonyl-fluoride, 0.02% NaN₃, in PBS. The same blocking solution was used in the following steps as incubation medium for the antibodies or as washing solution. Immunodetection of the antigens bound to the nitrocellulose membrane was then achieved by a double antibody reaction: the membrane was first reacted with Mabs (0.1 to 5 μg/mL) for three hours, washed three times (10 minutes each), and incubated for one hour with 125I-labeled rabbit anti-mouse IgG antibodies. All the steps were performed at room temperature. The membrane was washed again three times with BLOTTO and once with PBS and was dried and exposed to x-ray films.

For the immunoblotting of HMWK in whole plasma, pooled NHP or individual plasma samples were diluted 1:5 in TBS-BSA; then 30-μL aliquots were mixed with an equal amount of sample buffer, boiled for five minutes, and applied to the gel.

For the development of a quantitative immunoblotting assay of HMWK in plasma, optimal conditions for the transfer of antigen were evaluated by measuring the recovery of 125I-labeled HMWK on the nitrocellulose membrane. Increasing amounts of 125I-HMWK, diluted in TBS-BSA or in plasma, were blotted onto nitrocellulose membranes at 5 V/cm, at transfer times of 1, 2, 3, and 4 hours. Sixty-five percent of the radioactivity applied to the gel could be recovered after a two-hour transfer, with a negligible increase after longer transfer times. In addition, the transfer was found to be proportional to the amount applied to the gel only when 125I-HMWK added to NHP was diluted in HMWK-deficient plasma, but not when it was diluted in TBS-BSA, indicating that a constant amount of carrier plasma protein was essential to obtain a proportional transfer. Therefore, the reference curve for the quantitative assay was prepared by mixing 1:5 diluted NHP with 1:5 diluted HMWK-deficient plasma to obtain values of 80%, 60%, 40%, and 20% NHP in a constant amount of total plasma (6 μL). Individual plasma samples were similarly assayed by mixing equal volumes of the test plasma and HMWK-deficient plasma. After immunoblotting and exposure to x-ray films was done, the radioactivity corresponding to HMWK bands was counted in strips cut from the nitrocellulose membranes.

Contact Activation of Plasma for Immunoblotting Experiments

To evaluate whether the immunoblotting technique could detect the specific cleavage of HMWK in plasma, the contact system was activated in vitro, using glass or kaolin. In the glass activation experiments, 200 μL of plasma was incubated in a 12 × 75-mm glass tube for 15 minutes at 37 °C. Plasma was then diluted 1:5 in TBS-BSA, and 30-μL aliquots were mixed with equal amounts of sample buffer, boiled, and electrophoresed. In the kaolin activation experiments, 180 μL of plasma was incubated with 20 μL of a 200 mg/mL of kaolin suspension in polystyrene tubes at 37 °C for 15 minutes. After centrifugation at 5,000 g for two minutes, the supernatant was removed, and the kaolin pellet was washed twice with 200 μL of TBS-BSA containing 50 μg/mL of SBTI to quench the reaction. Proteins were extracted from the kaolin pellet by adding 200 μL of a 10% SDS solution. Six-microliter aliquots of kaolin supernatant and kaolin extract were then mixed with sample buffer, electrophoresed, and subjected to immunoblotting.

RESULTS

Purification of HMWK

The purified HMWK had a specific activity of 16 clotting units per milligram and migrated as a single band of 110,000 apparent mol wt when analyzed in a continuous buffer SDS-PAGE system, either in the presence or absence of reducing agents (Fig 1a). However, when the same preparation was electrophoresed in a discontinuous buffer SDS-PAGE system (Fig 1b), Coomassie blue stained two distinct protein bands, a major band with an apparent mol wt of 120,000 and a minor one with a mol wt of 105,000. Under reducing conditions, the major band showed a slightly faster mobility (~110,000 mol wt). When eluted from replicate SDS gels, both bands had HMWK clotting activity (~10 clotting units per milligram for the 120,000-mol-wt band, and ~2 clotting units per milligram for the 105,000-mol-wt band) and were cleaved by purified human kallikrein to give characteristic heavy and light chains on reduced SDS gels (data not shown).

After prolonged (60 minutes) incubation of the purified HMWK with purified plasma kallikrein, two closely migrating bands were generated with ~100,000 and 95,000 mol wt on nonreduced SDS gels (Fig 2). In the presence of a reducing agent, both bands disappeared to produce cleavage fragments having apparent mol wts of 62,000 and 50,000. These last two polypeptide chains, termed heavy and light chains, were separated by ion-exchange chromatography after reduction and alkylation of the cleaved molecule. The 50,000-mol-wt fragment retained the original clotting activ-
Fig 2. Cleavage of purified high-mol-wt kininogen (HMWK) by purified human plasma kallikrein. Lane (A) contains 8 μg of nonreduced HMWK incubated with Tris buffered saline (TBS). Eight micrograms of HMWK were incubated with kallikrein (80 ng) at 37 °C for one hour and then electrophoresed in a 7.5% discontinuous buffer sodium dodecyl sulfate-polyacrylamide gel electrophoresis system, in the absence (B) or in the presence (C) of 6 mmol/L of dithiothreitol.

Fig 3. Immunoblotting of purified high-mol-wt kininogen (HMWK) and its cleavage derivatives using monoclonal antibodies (Mabs). One microgram of single-chain (1) or two-chain (2), kallikrein cleaved, HMWK, and 0.5 μg of the purified alkylated heavy chain (3) and light chain (4) were electrophoresed in a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis system and transferred to nitrocellulose membranes. For the immunodetection, each membrane was then reacted with different Mabs. The pattern shown on the left (A) was obtained with Mab H1D; the pattern on the right (B) was obtained with Mab HSA. Mabs H2D and H4D gave the same pattern as HSA.

Immunoblotting of HMWK in Plasma

Two Mabs, one (H5A) directed against the heavy chain region and one (H1D) directed against the light chain region of HMWK, were selected for further studies of the protein in plasma samples using the immunoblotting technique. Samples containing 6 μL of plasma were electrophoresed in a discontinuous buffer SDS-PAGE system, transferred to a nitrocellulose membrane, and then reacted with one of the two Mabs as described in the Materials and Methods section.

Mab H1D. Mab H1D detected principally two bands in normal human plasma (Fig 4) with apparent mol wts of ~120,000 and ~105,000, respectively, under nonreducing conditions (Fig 4, lane a). This same pattern was obtained with purified HMWK (Fig 3). Under reducing conditions, NHP (Fig 4, lane f) and purified single-chain HMWK showed two closely migrating bands of ~110,000 mol wt. An identical pattern was observed for normal plasma samples collected using different anticoagulants (sodium citrate or

Table 1. Characteristics of Six Anti-HMWK Monoclonal Antibodies

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<tr>
<th>Characteristic</th>
<th>H1D</th>
<th>H2D</th>
<th>H3C</th>
<th>H4D</th>
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<tr>
<td>Double-chain HMWK</td>
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HMWK, high-mol wt kininogen.
HMWK IMMUNOBLOTTING IN PLASMA

Fig 4. Immunoblotting of unactivated and activated human plasma using monoclonal antibody (Mab) HD against the light chain region of high-mol-wt kininogen. Samples 6 μL were electrophoresed in a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis discontinuous system, transferred to a nitrocellulose membrane, and reacted with HD Mab (6 μg/mL). The following samples were applied to the gel: normal human plasma (NHP), nonreduced (A) and reduced (F); nonreduced plasma from a patient with hereditary total kininogen deficiency (B); glass-activated NHP, nonreduced (C) and reduced (G); supernatant of kaolin-activated plasma, nonreduced (D) and reduced (H); kaolin pellet extract, nonreduced (E) and reduced (I). Glass and kaolin activation of plasma are discussed in the Materials and Methods section. Samples were reduced with 6 mmol/L of dithiothreitol.

Fig 5. Immunoblotting of high-mol-wt kininogen (HMWK) in plasma from a patient with hereditary angioedema (C1-inhibitor deficiency), during (B) and (C), and seven days later near the end of an attack of angioneurotic edema (D). In lane (A), 6 μL of NHP were run for comparison. Samples on left were not reduced, whereas samples on right were reduced with 6 mmol/L of dithiothreitol.

HMMK IMMUNOBLOTTING IN PLASMA

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HMMK IMMUNOBLOTTING IN PLASMA

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hemin or EDTA) with or without the inhibitors (benzamidine, SBTI, leupeptin). The same pattern was observed in frozen pooled plasma or in plasma freshly collected from single individuals. Neither of the two bands was detectable in the plasma sample from an individual with hereditary total kininogen deficiency (Fig 4, lane b). A faint third band, with an apparent mol wt of 140,000 to 150,000, was sometimes detected in the unreduced plasma samples (Fig 4, lanes a through d), and it disappeared under reducing conditions (Fig 4, lanes f through h). For some normal plasma samples, this third faint band was not observed. Control experiments (data not shown) demonstrated that this band did not require the addition of the Mab and was not present in a plasma sample artifically depleted of IgG. Because this band comigrated with a band that was labeled by 125I-protein A, it probably represented cross-reactivity of some batches of the 125I-rabbit anti-mouse IgG with intact human IgG.

When normal human plasma was exposed to glass for 15 minutes at 37 °C to initiate contact activation before SDS-PAGE, an immunoblotting pattern similar to that of the purified two-chain, cleaved kininogen was observed (Fig 4, lane c), with two close bands that disappeared under reducing conditions (Fig 4, lane g), indicating that during contact activation of plasma HMWK was cleaved. A faint band of 50,000 mol wt corresponding to the light chain was detected by the HD Mab under reducing conditions (Fig 4, lane g). The immunoblotting of the purified light chain of HMWK shown in Fig 3a, lane 4, was repeated under different experimental conditions. The signal detected in the autoradiographs was not modified when the same amount of light chain was pretreated with a reducing agent (6 mmol/L of DTT), but it was much weaker when the purified light chain was added to HMWK-deficient plasma, especially under reducing conditions. This experiment explains why the sensitivity of detection of the light chain of HMWK is decreased in reduced activated plasma samples.

Normal human plasma was also exposed to 20 mg/mL of kaolin, and the supernatant and kaolin-bound pellet were analyzed using immunoblotting techniques. As seen in Fig 4, most of HMWK originally present in NHP (lane a) was recovered from the kaolin pellet (Fig 4, lane e), with a small amount remaining in the kaolin supernatant (Fig 4, lane d). In both the kaolin extract and the kaolin supernatant, HMWK appeared as a two-chain molecule which, under reducing conditions (Fig 4, lanes h and i), dissociated to release the heavy chain (not detected by the monoclonal antibody used) and a light chain of 50,000 mol wt (Fig 4, lane i).

HMWK antigen in the plasma of a patient with a hereditary deficiency of C1 inhibitor was analyzed in plasma samples collected during and after an attack of angioedema (Fig 5). In the plasma sample collected from the patient on the first day of a severe attack of angioedema (Fig 5, lane b), HMWK was detected by the Mab HD as a two-chain, cleaved molecule which was not detected under reducing conditions, implying that cleavage had occurred in vivo. Twenty-four hours later, when the patient was spontaneously recovering (Fig 5, lane c), and seven days following the initial day of the angioedema attack, when the patient was essentially asymptomatic (Fig 5, lane d), single-chain HMWK was detected, indicating the reappearance in plasma of the intact, uncleaved molecule.

Mab HD. In addition to HMWK, the Mab HD directed against the heavy chain region of HMWK recognized two additional bands in normal human plasma, either activated or not, but not in plasma deficient in both LMWK and HMWK (Fig 6). The two bands, with mol wts of ~60,000 and ~54,000, respectively, should therefore correspond to LMWK, whose heavy chain shares antigenic determinants with the heavy chain of HMWK.

Quantitative Immunoblotting Assay of HMWK in Human Plasma

Using the same two Mabs (HD and HD) in an immunoblotting technique, we developed a specific and sensitive
Fig 6. Immunoblotting of plasma using anti-heavy chain monoclonal antibody H5A (1 µg/mL). Each lane contained 6 µL of nonreduced plasma: (A) normal human plasma (NHP); (B) plasma from a patient with hereditary total kininogen deficiency; (C) glass-activated NHP.

Fig 7. Quantitative immunoblotting of purified high-mol-wt kininogen (HMWK) in HMWK-deficient plasma. Varying amounts of HMWK were added to deficient plasma, electrophoresed, transferred to a nitrocellulose membrane, and finally reacted with either of the two Mabs. After autoradiography was performed, the radioactivity bound to the nitrocellulose at the two bands of HMWK was counted. A linear relationship was found between the amount of HMWK added to the gel and the radioactivity counted on the nitrocellulose membrane, and as little as 10 ng of HMWK were clearly visible in the autoradiograph and gave counts three times higher than the control sample of HMWK deficient plasma (Fig 7).

Similarly, in the immunoblotting of progressive dilutions of normal human plasma, the sensitivity of the technique was found to be ~3% of NHP (corresponding to 0.2 µL of plasma under our conditions). According to the immunoblotting technique, the concentration of HMWK in pooled normal plasma was ~55 µg/mL. Five replicate assays of plasmas from 17 male normal subjects gave a mean value of 100.7 ± 15.8% of pooled normal plasma with a mean interassay coefficient of variation of 15%. A good correlation (r = .78, N = 17, least-square regression) between the quantitative immunoblotting assay and the clotting assay of HMWK was also observed.

DISCUSSION

Since the first description of the immunoblotting of proteins,24 various applications and technical improvements have been reported (for review, see ref 26). In immunoblotting studies of a complex protein mixture such as plasma, the antibody probe used for the immunodetection of the antigen under investigation must be as specific as possible. Therefore, a series of murine hybridoma clones producing Mabs to human HMWK was prepared, and the purification and partial characterization of six Mabs is reported here. The specificity of these antibodies was first evaluated by a dot-immunobinding assay using the ascitic fluid and then by the immunoblotting of purified HMWK using the purified immunoglobulins. The specificity for kininogen of two Mabs (H1D and H5A) used here in the immunoblotting studies of plasma was confirmed by the observation that these antibodies did not recognize any protein in a plasma deficient in HMWK and LMWK. When the purified HMWK preparation used as immunogen to produce the murine monoclonal antibodies was analyzed in a discontinuous buffer SDS-PAGE system, two protein bands were detected, a major one with an apparent mol wt of 120,000 and a minor one with an apparent mol wt of 105,000. The question of whether the HMWK preparation contained a major contaminating protein that was not separated in the continuous buffer SDS-PAGE system was addressed by elution experiments. When eluted from unstained replicate gels, both the 120,000-mol-wt band and the 105,000-mol-wt band were able to shorten the prolonged clotting time of a HMWK deficient plasma, indicating that both contained HMWK clotting activity. In addition, the two bands were detected in normal plasma by both anti-light chain and anti-heavy chain HMWK Mabs, but not in LMWK- and HMWK-deficient plasma. Therefore, both the bands are HMWK. HMWK has been purified in several laboratories using different purification procedures and appeared as a homogeneous single-band protein when analyzed by a continuous phosphate-buffered SDS-PAGE system.27 In some reports, however, the puri-
fied protein was electrophoresed in a discontinuous buffer SDS-PAGE system; under these conditions, two distinct bands were identified.11,12 Mori and Nagasawa13 considered the lower band as a cleavage derivative of HMWK produced during the purification procedure. This hypothesis may or may not be true, because our results show the presence of two bands of HMWK in plasma freshly collected in the presence of serine-protease or cysteine-protease inhibitors. Pierce and Guimaraes28 and Thompson et al,11 on the other hand, suggested that human HMWK may be heterogeneous, and our finding that HMWK is directly detectable as two bands in immunoblots of plasma is in accord with this hypothesis. Moreover, it is possible that a second form of single-chain HMWK can be identified that comigrates with cleaved unreduced HMWK.

The in vitro cleavage of HMWK in plasma was studied using the immunoblotting technique with an anti-light chain Mab (H1D). In plasma subjected to contact activation by glass or kaolin, the unreduced cleaved HMWK was seen as two close bands near 100,000 apparent mol wt in a discontinuous SDS-PAGE system (Fig 4), and the immunoblotting of plasma essentially reproduced the pattern observed in SDS-gels of purified HMWK cleaved by purified human plasma kallikrein. Further evidence of HMWK cleavage was provided by the disappearance of the two bands in reduced plasma samples even if the cleavage fragment (the light chain of HMWK) was not always clearly detectable.

The ability of the immunoblotting technique to detect the in vitro cleavage of HMWK in plasma prompted us to evaluate whether the method was suitable for the detection of an in vivo cleavage of HMWK in pathologic conditions. In a preliminary study, we analyzed plasma samples from a patient with a hereditary deficiency of C1 inhibitor, a clinical condition characterized by recurrent attacks of mucocuta-

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REFERENCES

13. Mori K, Nagasawa S: Studies on human high molecular edema).28 The typical attacks have been generally attributed to the production of proinflammatory mediators derived from the complement cascade following the spontaneous and unimpeded activation of C1. However, a potential pathogenic role for kinin derived from the activation of the contact system has also been proposed.30-32 Suction-induced blister fluids from patients with hereditary angioedema contain active plasma kallikrein.31 In addition, peripheral blood collected during a typical angioedema attack contains decreased levels of plasma prekallikrein activity and of HMWK activity and antigen.32 Further evidence is now provided by immunoblotting studies of plasma which show that during a typical attack of angioedema HMWK circulates in plasma as a two-chain, cleaved molecule (Fig 5).

The immunoblotting of plasma with a Mab directed against the heavy chain region of HMWK also detected LMWK. The immunoblots suggest that two different forms of LMWK exist in human plasma (Fig 6). The presence of two or more distinct forms of LMWK has been demonstrated in rat plasma33 and has been suggested for human and bovine plasma.28,34

Immunoblotting studies presented here using Mabs allow the qualitative and quantitative investigation of HMWK in plasma. The results indicate that multiple molecular forms, both uncleaved and cleaved, can be identified in plasma without the need for purification or radiolabeling procedures. These immunoblotting techniques that monitor in vitro and in vivo cleavage of HMWK may be useful in obtaining new information on the structure and function of HMWK and its role in pathologic processes.

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Detection of in vitro and in vivo cleavage of high molecular weight kininogen in human plasma by immunoblotting with monoclonal antibodies

M Berrettini, B Lammle, T White, MJ Heeb, HP Schwarz, B Zuraw, J Curd and JH Griffin

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