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# Quantification of Human High-Molecular-Weight Kininogen (HMW-KGN) by Specific Hemagglutination Inhibition Reaction[1] (39887)

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Human plasma contains two species of kininogens which differ in molecular weight and susceptibility to hydrolysis by plasma kallikrein (1–3). High-molecular-weight kininogen (HMW-KGN), but not low-molecular-weight kininogen (LMW-KGN), is a good substrate for plasma kallikrein and participates in contact activation of clotting, fibrinolysis, and kinin release (4–8). Despite these biological and physicochemical differences, HMW-KGN and LMW-KGN share antigenic determinants (2). In the present experiments goat antibody to a mixture of purified LMW-KGN and HMW-KGN produced reactions of identity against normal plasma, HMW-KGN, and LMW-KGN in double agar diffusion. Adsorption of the antiserum with excess LMW-KGN removed all of the antibody common to both species of kininogens, but the adsorbed antibody still reacted with HMW-KGN in a sensitive hemagglutination assay. This anti-HMW-KGN serum was used to quantify HMW-KGN in plasma from several normal and kininogen-deficient individuals with the sensitive tanned red cell hemagglutination inhibition assay (12).

*Materials and methods.* Hexadimethrine bromide (Polybrene) and trisodium ethylenediamine tetraacetate (EDTA) were obtained from the Aldrich Chemical Company, Milwaukee, Wisconsin; DE-23 cellulose was obtained from H. Reeve Angel, Clifton, New Jersey; Centrolex-O phospholipid used for partial thromboplastin times was obtained from Central Soya, Chicago, Illinois, and kaolin (acid washed) used in the same procedure was obtained from Fisher Scientific Co., Cincinnati, Ohio; QAE-Sephadex A-50, Sephadex G-150, and CM-Sephadex C-50 were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey; crystalline salt-free trypsin was obtained from Worthington Chemicals Corp., Freehold, New Jersey, and soybean trypsin inhibitor and Tris from Sigma Chemicals, St. Louis, Missouri. Benzamidine hydrocloride was obtained from Matheson, Coleman and Bell, Norwood, Ohio, and agarose from Fisher Chemicals, Cincinnati, Ohio.

All plasmas were separated from blood drawn into 1/50th vol of 0.5 *M* sodium citrate buffer, pH 5.2, using silicone-coated syringes and vessels. Plasma was recentrifuged at 20,000*g* to remove the platelets and stored in small aliquots at −70°. Plasma deficient in HMW-KGN was from patient Reid (the gift of Dr. C. L. Lutcher, Veterans's Administration Hospital, Augusta, Ga.) and from Mr. Fitzgerald (the gift of Dr. Robert Waldmann, Henry Ford Hospital, Detroit, Mich.). Plasma from the patient deficient in both HMW-KGN and LMW-KGN has been described earlier (7).

*High-molecular-weight kininogen* was isolated from diluted normal human plasma by modifications of published procedures (5, 9–11) by adsorption and elution from DE-23, chromatography on QAE-Sephadex A-50, CM-Sephadex C-50, and gel filtration on a 2.5 × 80-cm column of G-150 Sephadex. The kininogen in fractions was identified by immunological reaction with monospecific antibody to kininogens, release of kinin when exposed to trypsin, and its capacity to shorten the clotting time of Fitzgerald trait plasma (6, 7). Trypsin in all

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digestion mixtures was neutralized with an excess of soybean trypsin inhibitor before assay for kinin activity. The final product released 10-$\mu$g equivalents of bradykinin/mg of protein and, upon analytical electrophoresis in 7% polyacrylamide gel at pH 9, it formed two closely associated bands, each of which contained antigenic properties of kininogens when eluates of a corresponding section of identical unstained gels were reacted with antiserum to kininogen in double agar diffusion.

*Low-molecular-weight kininogen* was isolated from normal human plasma by modifications of methods published earlier (5, 9–11) by the same procedure used to prepare HMW-KGN. The LMW-KGN in column fractions contained antigens which reacted with the antibody to kininogen, and released kinin upon exposure to trypsin, but did not contain Fitzgerald factor clotting activity. After tryptic digestion, 25 $\mu$g of kinin were released/mg of protein; kinin activity was quantified by comparing the time of onset of muscle contraction to a plot of times of contractions by solutions of weighed amounts of synthetic bradykinin (Aldrich Chemical Company, Milwaukee, Wis.). Analytical polyacrylamide disc gel electrophoresis revealed that the LMW-KGN was not homogeneous, but contained four bands which stained with Coomassie brilliant blue.

*Buffers* used in chromatographic procedures contained hexadimethrine bromide (Polybrene) at concentrations of 50 $\mu$g/ml, $10^{-3}$ *M* benzamidine, and $10^{-4}$ *M* EDTA.

Protein concentrations were determined by comparing the $A_{280}$ of test solutions with a plot of the $A_{280}$ of serial dilutions of a weighed solution of crystalline bovine serum albumin.

Human erythrocytes from group O–Rh negative outdated blood were used in the *hemagglutination* assay, performed by modifications of the method of Merskey and his associates (12). The cells were washed five times with 20 vol of 0.075 *M* phosphate buffer, pH 6.6, containing 0.075 *M* sodium chloride, and a 4% suspension was stirred gently in a solution of 0.0025% tannic acid in the same buffer at room temperature for 30 min. The tanned cells were washed three times with 20 vol of 0.075 *M* phosphate buffer containing 0.05 sodium citrate, pH 6.6, and a 4% suspension was then mixed with an equal volume of HMW-KGN in the pH 6.6 phosphate–citrate buffer or with the buffer alone (control cells), and incubated at 37° for 30 min. For each 1 ml of packed tanned cells, 30–40 $\mu$g of HMW-KGN was used. Coated and control cells were washed three times with 20 vol of the phosphate-citrate buffer and stored at 4° for use within 2 weeks, or were stored in 40% glycerol at −70° for longer times (12). Control cells could not be agglutinated by antikininogen sera, human plasma, nonimmune goat serum, kininogen preparations, or buffers.

*Goat antibody to human kininogens* was raised by injecting 1 mg of a mixture of HMW-KGN and LMW-KGN in an equal volume of complete Freund's adjuvant intramuscularly at weekly intervals until precipitating antibody was produced (4 weeks). The antibodies to proteins contaminating the preparations of kininogen were removed by adsorption with serum from a kininogen-deficient individual (7). Subsequent challenging immunizations contained 1 mg of HMW-KGN (0.5 to 1.0 ml) and an equal volume of complete Freund's adjuvant. The final antibody and its $\gamma$-globulin fractions, prepared by octanoic acid precipitation (13) and ammonium sulfate fractionation, gave a single band of identity reaction with normal human plasma and with preparations of HMW-KGN and LMW-KGN (Fig. 1). *Sheep antiserum to LMW-KGN,* generously supplied by Dr. J. V. Pierce, National Institutes of Health, gave a reaction of identity with the *goat* antiserum to HMW-KGN and LMW-KGN when both were reacted with normal human plasma.

*Hemagglutination inhibition* was measured by mixing 0.1 ml of antiserum with 0.1 ml of diluted plasma, or other source of kininogen antigen, in a plastic hemagglutination assay plate containing rounded wells 2 cm in diameter and 0.7 cm in depth, and agitating these mixtures on an Adams Nutator for 30 min at room temperature. Then, 0.1 ml of 0.075 *M* phosphate buffer (pH 6.6) in 0.05 *M* sodium citrate containing 0.1% bovine serum albumin and 0.03 ml of a 4% suspension of erythrocytes coated with

HMW-KGN was added to the antigen–antibody mixtures, agitated for 10 min more at room temperature, and the formation of visible red cell aggregates was observed. The endpoints in the titration were read as the highest dilution of antigen at which red cell agglutination was inhibited. Antibody and antigen were diluted in the pH 6.6 phosphate citrate buffer containing 0.1% bovine serum albumin. Plastic or silicone (GE-Dri-Film, General Electric Co., Waterford, N.Y.) coated glass was used throughout.

*Results and discussion.* Goat antiserum to both human HMW- and LMW-KGN agglutinated erythrocytes coated with HMW-KGN at a titer of 5120. When it was adsorbed with 1.0 mg/ml of purified HMW-KGN the agglutination titer of the antiserum was reduced to less than 1, but adsorption with 0.5 to 1.2 mg/ml of LMW-KGN reduced the titer only to about 500 (Fig. 2a). Therefore, this antiserum appeared to contain antibodies to antigenic determinants unique to HMW-KGN as well as some to determinants common to both HMW and LMW-KGNS. When the goat antiserum had been adsorbed with excessive amounts of LMW-KGN to remove antibodies common

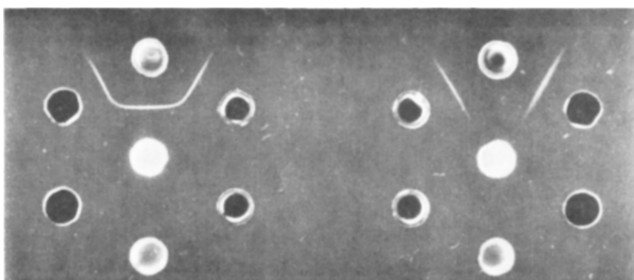

FIG. 1. The wells, numbered clockwise from the top, contain: (1) Monospecific goat antiserum to HMW-KGN and LMW-KGN; (2) LMW-KGN; (3) HMW-KGN; (4) Monospecific goat anti-kininogen serum absorbed with LMW-KGN (anti-HMW-KGN); (5) LMW-KGN; (6) HMW-KGN. The center well on the left contains normal human plasma; the center well on the right contains kininogen-deficient plasma (7).

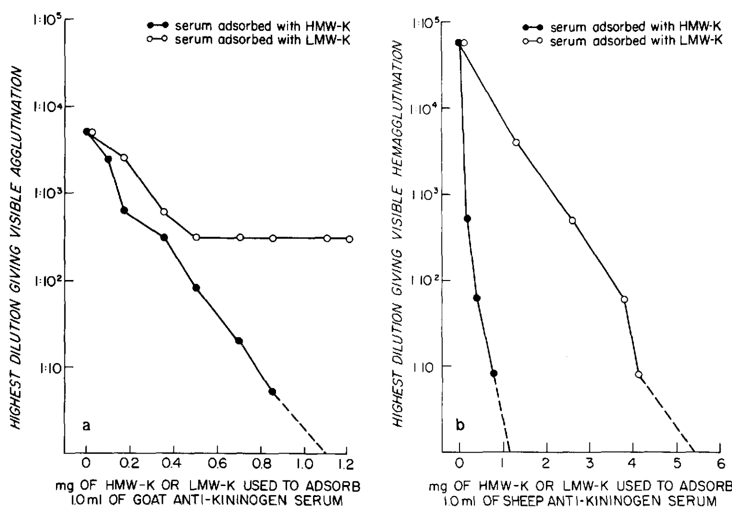


FIG. 2. (a) Monospecific goat antiserum was incubated with varying amounts of purified preparations of HMW-KGN or LMW-KGN at 37° for 1 hr and at 4° for 18 h. After the immune precipitates were removed by centrifugation each adsorbed antiserum sample was serially diluted with phosphate–citrate buffer containing bovine serum albumin, and 0.2 ml of each solution was then added with 0.1 ml of phosphate–citrate buffer and 0.03 ml of tanned erythrocytes coated with HMW-KGN, and hemagglutination reaction was allowed to proceed. (b) Monospecific sheep antiserum directed against LMW-KGN was adsorbed as above and the hemagglutination reaction was then measured.

to both forms of kininogen, it was designated anti-HMW-KGN.

The titer of a sheep antiserum to LMW-KGN was reduced from 60,000 to less than 1 by adsorption with 1 mg of HMW-KGN/ml or with about 5 mg of LMW-KGN/ml (Fig. 2b), as if this antiserum contained antibodies only to antigenic determinants shared by both HMW- and LMW-KGNS. This antiserum contained a much higher titer of antibody than the goat antiserum. The fact that about five times as much LMW-KGN as HMW-KGN was required to neutralize antibody in the sheep antiserum is probably due to the greater degree of purification of the HMW-KGN as compared to the LMW-KGN used. The LMW-KGN preparation appeared to contain more bradykinin than the HMW-KGN. Losses of this peptide during preparation of the HMW-KGN have been significant, but less bradykinin is lost during preparation of the LMW-KGN.

The titer of inhibition of hemagglutination with anti-HMW-KGN by normal plasma was 64 (Table I). The inhibition titer of plasmas from persons with hereditary deficiency of only HMW-KGN (Nos. 1 and 2, Table I) or with a deficiency of all forms of kininogen (7) was less than 1 (Table I). When the titers were measured using anti-LMW-KGN, however, the plasma deficient only in HMW-KGN was clearly distinguished from that deficient in all species of kininogen (Table I, column 3).

The titer of inhibition of hemagglutination of a sample of diluted normal plasma was 32; when an equal volume of a preparation of HMW-KGN with a titer of 32 was mixed with the plasma, the titer of the mixture was 64 (Table II). The same HMW-KGN increased the hemagglutination inhibition titer of plasma deficient in both kininogens from less than 1 to 32 (Table II).

Plasmas from unaffected and partially deficient (heterozygous) members of a kindred with kininogen deficiency were tested for their capacity to inhibit hemagglutination with anti-HMW-KGN. Those who were partially deficient in total plasma kininogens (7) were proportionally deficient in HMW-KGN antigens (Table III, columns 2 and 3) and the amount of HMW-KGN antigen was proportional to the amount of Fitzgerald factor clotting activity in those plasmas (Table III, column 4).

TABLE I. PLASMA TITERS OF INHIBITION OF HEMAGGLUTINATING ACTIVITY OF ANTI-HMW-KGN AND ANTI-LMW-KGN.[a]

| Plasma | Titer[b] of Activity vs | |
|---|---|---|
| | Anti HMW-KGN | Anti LMW-KGN |
| Normal | 64 | 128 |
| HMW-KGN deficient No. 1 | <1 | 64 |
| HWM-KGN deficient No. 2 | <1 | 64 |
| KGN deficient[c] | <1 | <1 |

[a] One-tenth milliliter of a 1/10 dilution of monospecific goat anti-HMW-KGN or of a 1/1600 dilution of monospecific sheep anti-LMW-KGN was mixed with 0.1 ml of serial dilutions of plasma, and the hemagglutination inhibition reaction was measured as described in Methods. Plasmas were serially diluted from 1/1 to 1/512 in each assay.

[b] Reciprocal of highest dilution of plasma at which hemagglutination was blocked.

[c] Deficient in all species of plasma kininogens (7).

TABLE II. TITER OF HEMAGGLUTINATION INHIBITION BY HMW-KGN.[a]

| Antigen mixture (equal parts) | Titer |
|---|---|
| NHP + buffer | 32 |
| KGN-deficient plasma + buffer | <1 |
| KGN-deficient plasma + HMW-KGN | 32 |
| HMW-KGN + buffer | 32 |
| NHP + HMW-KGN | 64 |

[a] Normal plasma, kininogen-deficient plasma, or phosphate-citrate buffer with0.1% bovine albumin were each mixed with the HMW-KGN preparation (0.1 mg/ml), serially diluted, and 0.1 ml of each dilution was mixed with 0.1 ml of monospecific goat anti-HMW-KGN serum diluted 1/10. Then the hemagglutination inhibition test was performed as described. The titer of hemagglutination is the reciprocal of the highest dilution of the plasma mixtures at which hemagglutination was completely inhibited.

To determine the amount of HMW-KGN in a pool of 25 normal human plasmas, the titer of hemagglutination inhibition was compared to that of dilution of a solution of HMW-KGN containing a known amount of HMW-KGN protein. The concentration of HMW-KGN in this plasma pool was 90 $\mu$g/ml. The variability of plasma HMW-KGN concentration in health and disease is to be determined.

The goat antiserum to kininogens used in these experiments was rendered monospecific for kininogens by adsorption with plasma from an individual with a severe in-

TABLE III. HMW-KGN ANTIGEN AND CLOTTING ACTIVITY IN PLASMAS FROM MEMBERS OF A KGN-DEFICIENT KINDRED.[a]

| | | Percentage of normal | |
|---|---|---|---|
| Plasma | Hemagglutination inhibition titer[b] | Hemagglutination inhibition | Clotting activity[c] |
| Pool of 25 normal | 60 | 100 | 100 |
| K.M. | 50 | 83 | 84 |
| I.W. | 30 | 50 | 50 |
| S.W. | 30 | 50 | 45 |
| M.M. | 90 | 150 | 150 |
| D.W. | 40 | 66 | 46 |
| V.T. | 30 | 50 | 60 |

[a] The capacity of plasmas from partially deficient and unaffected members of a kindred (7) with hereditary kininogen deficiency to neutralize anti-HMW-KGN was measured as in the experiments shown in the previous tables, but utilizing a different dilution series, noted above. In the left column, the titer of hemagglutination inhibition is given as the reciprocal of the highest dilution of the plasma at which hemagglutination was completely blocked. In the center column, the percentage of Fitzgerald factor clotting activity is given. The percentage of clotting activity was derived by comparing clotting times of dilutions of test plasma to a double-logarithmic plot of clotting times of dilutions of normal plasma used in the assay for Fitzgerald factor activity (6, 7). The percentage of hemagglutination inhibition was estimated arithmetically from the highest dilution of normal plasma which completely *inhibited* agglutination (1/60).

[b] Ten dilutions from 1/10 through 1/100 tested at dilution differences of 1/10.

[c] Measured as Fitzgerald factor activity (see Methods).

herited deficiency of all species of kininogen molecules (7). It was conceivable that this antiserum still contained traces of antibody directed against other plasma proteins. The fact that plasma from persons deficient only in HMW-KGN (Table I) but not in LMW-KGN lacking Fitzgerald factor clotting activity did not block the hemagglutination reaction using the anti-HMW-KGN excludes this possibility. In addition, plasma from heterozygous members of a kindred with partial deficiencies of HMW-KGN, LMW-KGN, and Fitzgerald factor clotting activity (7) was partially deficient in HMW-KGN in the hemagglutination inhibition assay (Table III). In other studies, the levels of plasma kininogen in the heterozygous individuals were found to be decreased using an electroimmunodiffusion technique.

These observations indicate that there are some antigenic determinants on HMW-KGN molecules distinct from those found on LMW-KGN molecules, and some antigenic determinants common to both molecules (2). The anti-HMW-KGN serum adsorbed with LMW-KGN did not provide a visible precipitin reaction in double agar diffusion. It is possible that the antibody is not a precipitating antibody, or, alternatively, that the quantity of the precipitin reaction obtained is too small to be visible. Such an antiserum will be of practical value in quantifying the amounts of HMW-KGN in body fluids.

*Summary.* HMW-KGN bears antigenic determinants shared by LMW-KGN, but also has antigenic properties unique to HMW-KGN. An antiserum which reacted only with HMW-KGN was prepared by exhaustive adsorption of antiserum to both HMW-KGN and LMW-KGN with a preparation of LMW-KGN. Using such an antiserum, HMW-KGN can be quantified by a hemagglutination inhibition assay.

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