SUMMARY Monoclonal antibodies to purified human urinary kallikrein have been developed. Selection of antibody producing clones was based on $^{125}$I-kallikrein binding activity of hybridoma media in both radioimmunoassay and enzyme-linked immunosorbent assay. Three clones (2 IgG1, 1 IgG2b) were subcloned, characterized, and compared with the polyclonal antiserum generated in rabbits immunized with the purified kallikrein. With radioimmunoassay, mouse ascitic fluids or rabbit antisera dilutions showing 50% binding to $^{125}$I-kallikrein were $1:1.2 \times 10^6$ (E7A9), $1:1.2 \times 10^5$ (H6A6), $1:8.0 \times 10^4$ (E12H1), and $1:1.4 \times 10^5$ (the rabbit antisera). With enzyme-linked immunosorbent assay, mouse ascitic fluids from clones E7A9 and H6A6 showed half-maximal absorbance at dilutions of $1:2.1 \times 10^5$ and $1:1.0 \times 10^5$ respectively, and the polyclonal antiserum showed half-maximal absorbance at a dilution of $1:2.0 \times 10^6$. These monoclonal antibodies showed no cross-reactivity with rat tissue kallikrein, rat urinary plasminogen activator, or dog pancreatic kallikrein, while the polyclonal antiserum showed some cross-reactivity. The binding of monoclonal or polyclonal antibodies to $^{125}$I-human urinary kallikrein was not affected by human plasma kallikrein, thrombin, or urokinase in a competitive radioimmunoassay. By using purified human urinary kallikrein immobilized to agarose, antibodies produced by clones E7A9 and H6A6 and in the rabbit antisera were purified to homogeneity. Each of these affinity-purified antibodies inhibited the esterase activity, and two of the three inhibited the kininogenase activity, of human urinary kallikrein. A sandwich immunosorbent assay was developed to measure this kallikrein using monoclonal antibody from the clone E7A9 in conjunction with the polyclonal antibodies. The sensitivity of this assay was 80 pg per well (800 pg/ml) of human tissue kallikrein, which was measured in urine, saliva, or pancreatic juice. Western blot analysis with affinity-purified polyclonal antibodies identified tissue kallikreins in human seminal fluid, saliva, and pancreatic juice. These results show that both a monoclonal antibody and affinity-purified polyclonal antibodies to human urinary kallikrein recognize determinants that affect enzyme activity, and they can be used for enzyme-linked immunosorbent assay quantitation and perhaps as specific inhibitors of human tissue kallikrein. (Hypertension 7: 931-937, 1985)
In this study, we have developed and characterized three mouse hybridomas producing monoclonal antibodies to human tissue kallikrein. We found no cross-reactivity of monoclonal antibodies with the available purified human serine proteases: plasma kallikrein, thrombin, or urokinase in a competitive binding assay. Also, there was no cross-reactivity with rat tissue kallikrein, rat urinary plasminogen activator, or dog pancreatic kallikrein. Two of the antibodies are inhibitory, while one appears to stimulate the kininogenase activity of purified human urinary kallikrein. By using polyclonal antibodies in conjunction with monoclonal antibodies, we have also developed a sandwich immunosorbent assay for measuring human tissue kallikrein in human body fluids.

Materials and Methods

Purification of Kallikreins and Antibodies

Human urinary kallikrein was purified to homogeneity and polyclonal antisera generated in rabbits as described previously. Rat urinary kallikrein, rat urinary plasminogen activator (esterase A), and dog urinary and pancreatic kallikreins were purified according to previously described procedures. Affinity purified human urinary kallikrein (2 mg/ml in 0.1 M 4-morpholinopropane sulfonic acid, pH 7.3) was mixed with 25 ml of Affi-Gel 15 (Bio-Rad, Rockville Center, NY, USA) that had been prewashed with distilled water on a sintered glass filter. Incubation was performed with gentle shaking for 4 hours at room temperature and then for 16 hours at 4 °C. One-tenth volume of 1 M ethanolamine-HCl (pH 8.0) was added to block the remaining unreacted sites. Following incubation for 1 hour at 23 °C, the conjugate was washed on a Whatman No. 40 filter paper (Clifton, NJ, USA) several times with distilled H2O. Kallikrein-agarose was then equilibrated with 0.1 M NaCl, 0.01 M sodium phosphate, pH 7.0, and used for purification of polyclonal antisera or monoclonal ascitic fluids as described previously. Rabbit IgG from normal rabbit serum was purified using protein A-sepharose affinity chromatography. Purified kallikreins or protein A was labeled with 125I using the lactoperoxidase method according to a protocol described by Shimamoto et al. Purified human plasma kallikrein was provided by Dr. Allen P. Kaplan, human thrombin by Dr. Alan D. Cardin, and human urokinase by Dr. Kazuaki Shimamoto.

Hybridoma Development

Six-week-old female BALB/c mice were immunologically primed with an intraperitoneal injection of 50 μg of purified human urinary kallikrein emulsified in complete Freund’s adjuvant. Three weeks later the mice were boosted with 50 μg of enzyme in incomplete Freund’s adjuvant. Four days later, spleen cells were obtained from the mice. The cells were then mixed with Sp2/O-Ag 14 mouse myeloma cells (Dr. Hyman, Salk Institute, San Diego, CA, USA) in a 4:1 ratio and fused with 50% polyethylene glycol-4000 (Fisher Scientific Co., Fairlawn, NJ, USA). Hybrids were selected with hypoxanthine-aminopterine-thymidine medium. When clones became visible, the supernatant (0.1 ml) was collected and kallikrein antibody was measured with a radioimmunoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA). Clones producing antibody were subcloned by limited dilution with 0.5 cell plated per well, and ascitic fluid was produced in syngenic mice. Monoclonality was also assessed for each of these clones by using subclass-specific anti-mouse immunoglobulin in an ELISA, which resulted in only one subtype being identified for each of the clones tested. Daily visual inspection of each of the subclonal wells, macroscopically as well as microscopically, showed only one clone visible in a well with hybrid growth.

A microtiter plate assay was developed according to standard procedures. Polyvinyl microtiter plates (Falcon Becton Dickinson, Oxnard, CA, USA) were coated with 0.5 μg/well of purified human urinary kallikrein in 0.1 ml of phosphate-buffered saline (PBS) for 2 to 3 hours at 37 °C. The plates were blocked with 5% bovine serum albumin in PBS for 1 hour at 37 °C and then washed with wash buffer (PBS containing 0.05% polysorbate 20 [Tween 20]). Fluids containing the primary antibody (i.e., antisera, hybridoma media, purified monoclonal antibodies, or rabbit anti-mouse immunoglobulin subclasses; Cappel Laboratories, West Chester, PA, USA) were serially diluted in PBS containing 1% bovine serum albumin and 0.05% Tween 20. Aliquots (100 μl) were applied to each well and incubated at 37 °C for 90 minutes. The wells were washed as before with wash buffer, and 10 μl of peroxidase-conjugated goat anti-mouse IgG (1:500; Cappel) in PBS containing 1% bovine serum albumin was allowed to bind for 1 hour at room temperature. The unbound antibody was removed by washing plates seven times with the wash buffer. The specifically bound peroxidase-conjugated antibodies were detected by the formation of a color reaction produced when freshly prepared substrate solution (0.03% 2,2’-azino-di(3-ethylbenzthiazoline sulfonic acid) and 0.003% H2O2 in 0.05 M citrate buffer, pH 4.0) was added to each well. The color reaction was read after 30 minutes at 414 nm in a Titertek Multiscan (Flow Laboratories, Inc., McLean, VA, USA).

Microtiter plates were coated with 100 μl/well of affinity-purified rabbit anti-kallikrein antibodies (2 μg/ml in carbonate buffer, pH 9.6) overnight at 4 °C and blocked as already described. Human urinary kallikrein and samples of human saliva, pancreatic juice, or urine were diluted in PBS containing 1% bovine serum albumin and 0.02% Tween 20. Kallikrein in both the standards and the samples was initially bound to a monoclonal reagent (for increased sensitivity) by preincubation with an ascitic fluid (1:10,000) for 1 hour at 37 °C. The kallikrein—monoclonal antibody complex in the preincubated samples (100 μl) was next incubated for 90 minutes at 37 °C with affinity-purified rabbit anti-kallikrein antibodies that had been immobilized on microtiter plates. The unbound material was then removed from the plate with four washings in the
wash buffer. Peroxidase-conjugated sheep anti-mouse IgG (1:2000 in PBS containing 1% bovine serum albumin) was then added and incubated for 1 hour at room temperature. The unbound antibody was removed by washing seven times with PBS containing 0.02% Tween 20, and the bound peroxidase conjugated antibodies were detected as already described. The dilution buffer or the monoclonal ascitic fluid (1:10,000) in the absence of the sample was used as a control.

Kallikreinlike Nω-tosyl-γ-arginine methyl ester (Tos-Arg-OMe) esterase activity and kininogenase activity were determined as described previously. The direct radioimmunoassay of tissue kallikrein was performed using the rabbit anti-human urinary kallikrein antiserum.

Electrophoresis was performed in a 7.5 to 17.5% linear gradient polyacrylamide gel containing 0.1% sodium dodecyl sulfate as described previously. The proteins were then electrophoretically transferred to nitrocellulose, as described by Burnette. Kallikreins were visualized by autoradiography with 125I-protein A binding.

Results

Hybridoma Development

Three separate fusions yielded approximately 30% growth of hybrids after 2 weeks. In the preliminary screening, hybridoma supernatants were tested for binding to human urinary kallikrein in RIA. Clones (~1% secreting kallikrein antibody) were selected for expansion. Three hybridoma clones secreting anti-human tissue kallikrein antibody were chosen based on the binding of crude hybridoma media to purified kallikrein in both RIA and ELISA. Each hybridoma was subcloned by limited dilution. The immunoglobulin subclass of each clone was determined by ELISA using specific antisera to each immunoglobulin subtype. The selected clones were designated E7A9 (IgG1), H6A6 (IgG2b), and E12H1 (IgG1). Monoclonal antibody reagents were prepared from ammonium sulfate-fractionated culture media and ascitic fluid. Each of these clones has been stable for 4 to 5 months in culture without loss of antibody production, as well as in liquid nitrogen storage with subsequent growth and antibody production after thawing.

Binding Properties

Figure 1 shows titration curves for each of the monoclonal ascitic fluids and the polyclonal antiserum as analyzed by RIA. Dilutions in a range from 1:400 to 1:4,000,000 showed concentration-dependent kallikrein binding activity. The dilutions showing 50% binding to 125I-kallikrein were $1.2 \times 10^6$ (E7A9), $1.2 \times 10^6$ (H6A6), $1.8 \times 10^6$ (E12H1), and $1.4 \times 10^6$ (polyclonal antiserum). Clone E7A9 showed a binding titer comparable to that of the polyclonal antiserum. The antibody titration curves of two of the monoclonal antibodies and the antiserum were also measured in an ELISA. Ascitic fluids produced by clones E7A9 and H6A6 showed half-maximal absorbance at dilutions of $1.21 \times 10^5$ and $1.10 \times 10^5$, respectively, compared with $1.20 \times 10^6$ for the polyclonal antiserum (Table 1). The nonspecific binding or absorbance of the tested fluids was approximately 5 to 10% of the total binding or absorbance.

Molecular Properties

Monoclonal and polyclonal antibodies were purified to homogeneity using affinity chromatography with purified urinary kallikrein immobilized to agarose (Figure 2). The relative molecular weights of the immunoglobulin heavy and light chains are shown in Table 1. None of the antibodies reacted with more than one mouse subclass-specific goat antiserum, as expected for hybridoma clones producing a single type of antibody. The reactivities of rat urinary kallikrein, rat urinary plasminogen activator (esterase A), and dog pancreatic or urinary kallikrein to polyclonal and
Table 1. Characterization of Anti-human Tissue Kallikrein Monoclonal and Polyclonal Antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Ig subclass</th>
<th>( M_r (\times 10^3) )</th>
<th>Binding titer</th>
<th>Enzyme inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Light chain</td>
<td>Heavy chain</td>
<td>RIA</td>
</tr>
<tr>
<td>Monoclonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E7A9</td>
<td>IgGl</td>
<td>28</td>
<td>56</td>
<td>1.2 x 10^6</td>
</tr>
<tr>
<td>H6A6</td>
<td>IgG2b</td>
<td>27</td>
<td>58, 62</td>
<td>1.2 x 10^5</td>
</tr>
<tr>
<td>E12H1</td>
<td>IgGl</td>
<td>28, 29</td>
<td>58</td>
<td>8.0 x 10^4</td>
</tr>
<tr>
<td>Polyclone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>—</td>
<td>31</td>
<td>53, 56</td>
<td>1.4 x 10^5</td>
</tr>
</tbody>
</table>

RIA = radioimmunoassay; ELISA = enzyme-linked immunosorbent assay.
* Determined by ELISA with monospecific antiserum against each subtype.
† Relative molecular weight of kallikrein-affinity-purified antibodies determined from sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
§ Binding titer expressed as the reciprocal of final dilution of monoclonal ascitic fluid or rabbit antiserum required to bind 50% of total \(^{125}\text{I}-\text{kallikrein} in RIA or giving half-maximal absorbance (1.0 unit) at \( A_{415} \) in an ELISA after a 30-minute incubation at room temperature.

Monoclonal antibodies against human urinary kallikrein were tested by either RIA or ELISA. Table 2 shows that polyclonal antiserum cross-reacted with kallikreins from other species and rat urinary esterase A, while monoclonal antibodies did not cross-react with any of the related enzymes.

**Effects of Antibody on Enzymatic Activity**

The effects of affinity-purified antibodies on kallikrein activity were measured using Tos-Arg-OMe or purified bovine low molecular weight kininogen as substrates (Figure 3). Kininogenase activity is expressed as the percent of kinin released in the absence of antibody. Antibodies from clones E7A9 and H6A6, as well as the polyclonal antibody, showed concentration-dependent inhibition. In contrast, antibody from

![Figure 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified antibodies. Antibodies (~6 µg each) were electrophoresed in a 7.5 to 17.5% linear gradient polyacrylamide gel under reducing conditions. The protein bands were visualized with Coomassie blue staining. Lane a, molecular weight markers: phosphorylase b (94,000); bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); and α-lactalbumin (14,400). Lane b, protein A-affinity purified normal rabbit IgG. Lane c, kallikrein-affinity purified rabbit anti-kallikrein antibody. Lanes d and e, kallikrein affinity-purified monoclonal antibodies E7A9 and H6A6 respectively.](http://hyper.ahajournals.org/)
clonal E12H increased the kininogenase activity of the kallikrein approximately 30%. However, all of the antibodies inhibited Tos-Arg-OMe esterase activity at antibody concentrations ranging from $2 \times 10^{-7}$ M to $2 \times 10^{-8}$ M (Table 1).

Quantitation of Human Tissue Kallikrein with Radioimmunoassay and Enzyme-Linked Immunosorbent Assay

Unlabeled human urinary kallikrein (250 pg–16 ng) competed with the binding of the monoclonal antibody E7A9 ($1.6 \times 10^6$) to $^{125}$I-kallikrein in a competitive binding assay (Figure 4). The binding of monoclonal antibodies H6A6 ($1.6 \times 10^5$), and E12H1 ($1.0 \times 10^5$) to $^{125}$I-kallikrein required higher concentrations of human urinary kallikrein (2–16 ng). In this competitive binding assay system neither the binding of monoclonal antibody E7A9 nor the polyclonal antiserum to $^{125}$I-human urinary kallikrein was affected by large amounts of human plasma kallikrein (5 μg/tube), human thrombin (10 μg/tube), or human urokinase (10 μg/tube).

Greater sensitivity, however, was achieved with E7A9 (1:10,000) when used in conjunction with the polyclonal antibodies to develop a sandwich immunosorbent assay. Figure 5 shows typical curves for the human urinary kallikrein, urine, saliva, and pancreatic juice. This assay allows the detection of immunoreactive kallikrein in the range from 80 pg to 1.25 ng per well. The quantity of immunoreactive human tissue kallikrein measured by ELISA in the random fluid collections was 125 ng/ml in urine, 563 ng/ml in saliva, and 688 ng/ml in the pancreatic juice.

Western Blot Analyses

To demonstrate the specific recognition of tissue kallikrein by affinity-purified polyclonal antibodies, human seminal fluid, saliva, and pancreatic juice was electrophoresed on a sodium dodecyl sulfate–poly-
FIGURE 5. Enzyme-linked immunosorbent assay of kallikrein in biological fluids with monoclonal antibody E7A9. Purified human urinary kallikrein (filled circles), human urine (triangles), saliva (squares), and pancreatic juice (open circles) were diluted and preincubated with anti-human urinary kallikrein monoclonal antibody (E7A9) ascitic fluid (final dilution, 1:10,000) for 1 hour at 37 °C. One-tenth milliliter of this mixture was added to microtiter plates precoated with 2 µg/ml of affinity-purified rabbit anti-human urinary kallikrein. Free monoclonal antibody was removed by washing four times with phosphate-buffered saline containing 0.05% Tween 20. Affinity-purified sheep anti-mouse IgG-horseradish peroxidase conjugate (1:2000) was added, and A414 was determined after the addition of 0.003% of H2O2 and 0.03% 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) in 0.05 M citrate buffer, pH 4, and incubation for 30 minutes.

FIGURE 6. Western blot analysis of human body fluids by affinity-purified rabbit anti-human urinary kallikrein antibodies and 125I-protein A. Human seminal fluid (60 µl), mixed whole saliva (60 µl), umbilical artery extract (60 µl, 4 mg/ml), human urinary kallikrein (4 µg), and pancreatic juice (60 µl) were electrophoresed on a linear gradient (7.5-17.5%) polyacrylamide gel containing 0.1% sodium dodecyl sulfate under reducing conditions. The proteins were electrophoretically transferred onto nitrocellulose paper. The transfer was incubated with rabbit anti-human urinary kallikrein antibody followed by 125I-protein A, and the binding was visualized by autoradiography. Lane a, seminal fluid; lane b, saliva; lane c, umbilical artery extract; lanes d and f, urinary kallikrein; lane e, pancreatic juice.

Discussion

Three hybridomas secreting monoclonal antibodies to human urinary kallikrein were selected and characterized. The monoclonal and polyclonal antibodies differed from one another in subunit molecular weights, binding titer, cross-reactivities with other kallikrein-like enzymes, and effects on kallikrein enzymatic activities. One of these monoclonal antibodies was used with our affinity purified polyclonal antibodies to develop a sensitive ELISA for the measurement of human tissue kallikrein levels in body fluids without the need of radioactive tracer enzyme.

The specificity of the monoclonal antibodies for human tissue kallikrein was illustrated by their lack of cross-reactivity with other available kallikreins or kallikrein-like enzymes from human, rat, or dog. The
polyclonal antibody to human tissue kallikrein, on the other hand, cross-reacted to some extent with other kallikreins and kallikrein-related enzymes. The results are consistent with the fact that kallikreins belong to a group of structurally and immunologically closely related arginyl esteropeptidases.

Titration curves are indicative of the concentration and binding strength of an antibody. Comparisons were made between monoclonal ascitic fluid and polyclonal antiserum. Polyclonal antiserum is known to be very potent, maintaining 50% binding titers to 125I-kallikrein at a dilution of 1:1.4 × 10^6. The binding titer from the ascitic fluid of the clone E7A9 was comparable to that of the polyclonal antiserum: it demonstrated 50% binding to 125I-kallikrein at a dilution of 1:1.2 × 10^4. Ascitic fluid from two other clones, however, showed weaker binding titers of 1:1.2 × 10^8 (H6A0) and 1:8.0 × 10^4 (Ei2H). Contrastingly, the binding titers obtained from an ELISA showed that clones E7A9 and H6A0 have binding titers higher than that of the polyclonal antisera.

The inconsistency between the binding values in RIA and ELISA is not unique to these studies with human urinary kallikrein monoclonal and polyclonal antibodies. They may result because the two assays are based on two separate and different physical principles. Our RIA is a liquid-phase assay that depends on an immune complex formation in solution with sufficient binding to allow precipitation by polyethylene glycol. The ELISA, on the other hand, is a solid-phase assay in which the antibody is required to bind an antigen that has been immobilized onto a solid surface. It is likely that at least some of the differences in binding are due to the immobilization of the antigen. The orientation of antigen immobilization to a surface depends on the surface topology and charge distribution of a given antigen, which will to some extent restrict the number of possible orientations of immobilization. Once immobilization has occurred, the epitopes bound by antibodies can be oriented in a manner that masks epitopes and/or sterically hinders antibody binding, and thus lowers binding values. Alternatively, the orientation of the epitopes could be such that they are more accessible for antibody binding and thus increase the binding values.

Evaluation of the effects of antibodies on enzymatic activity provides some information about possible antibody binding sites, as well as the potential usefulness of antibody as a specific reagent to manipulate enzymatic activity. Purified monoclonal antibodies from clones E7A9 and H6A0, as well as the rabbit antiserum, inhibited kininogenase activity in a dose-dependent manner, while the antibody from clone Ei2H appeared to stimulate the kininogenase, but not the esterase, activity of kallikrein. The results suggest that antibodies from clone E7A9 or H6A0 bind to some determinant at or near the active site of the enzyme molecule. The inhibition of enzyme activity by the polyclonal antibodies is not surprising, since numerous binding sites must be available to this heterogeneous population of antibodies, resulting in obstruction of functionally important sites or even precipitation of the enzyme. Of interest is the possibility that the antibody from clone E7A9 is a reagent capable of stimulating kallikrein activity in cellular populations or sites where kallikrein is present but the functional responsibilities of its product(s) is unclear. Experiments are in progress to determine whether this antibody will be useful in this regard.

Finally, this study shows that the coupled use of the polyclonal antibodies and a monoclonal antibody can result in an immunosorbent assay for tissue kallikrein of reasonable sensitivity without the necessity of 125I-labeled enzyme. It seems clear from the Western blotting analysis, however, that immunoreactive kallikreinlike material is present in several different molecular weight forms depending on the origin of the evaluated fluid or tissue.

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