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Enzymes and Protein Markers

Characterization of Monoclonal Antibodies for Prostate-specific Antigen and Development of Highly Sensitive Free Prostate-specific Antigen Assays

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Background: **The recent elucidation of the importance of serological free prostate-specific antigen (PSA) in the diagnosis of prostate cancer has created a demand for immunoassays specific for free PSA.**

Methods: **We developed and characterized 11 monoclonal antibodies with high affinities for PSA (K_a values from 1.1×10^8 to 1.8×10^{10} L/mol), only 3 of which cross-react with human glandular kallikrein (hK2). Using these antibodies and PSA antibodies developed by others, in conjunction with time-resolved fluorometry, we developed ultrasensitive sandwich immunoassays specific for the free form of PSA.**

Results: **The analytical detection limit of these immunoassays is 0.001 μg/L. To our knowledge, this is the most sensitive free PSA assay reported to date. The free PSA immunoassays exhibit <1% cross-reactivity with PSA-α_1-antichymotrypsin, show no cross-reactivity with hK2, and correlate well with established free PSA kits. The 11 antibodies developed by our group, in conjunction with 4 commercially available antibodies, were used to generate a putative epitope map of the PSA molecule.**

Conclusion: **The highly sensitive free PSA immunoassays may be used for measuring PSA subfractions in female serum, an application currently impossible with other reported free PSA immunoassays.**

Prostate-specific antigen (PSA)[4] is a 33-kDa single-chain glycoprotein expressed predominantly by the epithelial cells lining the acini and ducts of the human prostate *(1)*. After PSA is secreted into the lumen of the prostate gland, it becomes a constituent of seminal fluid. Present at concentrations of 0.5–5 g/L *(2)*, PSA is one of the major proteins of seminal plasma. The prostatic function of PSA is to liquefy the sperm-entrapping seminal coagulum after ejaculation *(3)*. The seminal substrates of PSA, a serine protease with chymotrypsin-like specificity *(4)*, are the major structural constituents of the gel-like coagulum, semenogelin I and II, and fibronectin. These proteins are degraded into smaller, soluble fragments by PSA, thereby increasing spermatozoa motility *(5)*.

PSA usually is released into the blood at low concentrations (<4 μg/L) in healthy males *(6)*. PSA forms stable complexes with two major extracellular serine protease inhibitors,α_1-antichymotrypsin (ACT) and α_2-macroglobulin *(7)*. PSA complexed to ACT is the predominant immunoreactive form of PSA in serum, whereas <30% of the total PSA occurs in the noncomplexed "free" form *(8, 9)*. The formation of complexes between PSA and α_2-macroglobulin masks all PSA epitopes, leaving no epitopes accessible for antibody recognition *(10)*; however, binding of ACT to the PSA molecule allows PSA antigenicity to be retained because several PSA epitopes remain unmasked *(9)*. Thus, PSA-ACT and free PSA are two molecular forms that may be monitored using immunological methods such as immunoassay.

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[4] Nonstandard abbreviations: PSA, prostate-specific antigen; ACT, α_1-antichymotrypsin; hK2, human glandular kallikrein; BSA, bovine serum albumin; DFP, diflunisal phosphate; SPR, surface plasmon resonance; and ISOBM, International Society for Oncodevelopmental Biology and Medicine.

Because of the correlation of PSA with tumor volume and tissue specificity *(11)*, its use as a tumor marker has flourished over the past decade. PSA immunoassays are widely used to detect early-stage prostate cancer, to evaluate disease progression, and to assess therapeutic response *(12)*. Furthermore, PSA concentrations may be used to identify postsurgical residual disease or tumor recurrence *(13)*. In addition to the total serum PSA concentration (free PSA plus PSA-ACT), the ratio of free to total PSA has become an important variable for distinguishing between males with benign and malignant prostatic disease. The percentage of free serum PSA is lower in males with prostatic carcinoma than in those with benign prostatic hyperplasia or with no apparent prostate pathology *(10, 14)*.

Despite the extensive use of PSA as a clinical marker of prostate cancer, much is yet to be learned about the antigenic determinants on the PSA molecule *(15)*. An understanding of the epitope distribution may yield insight into the antigenic characteristics of PSA and its interaction with anti-PSA antibodies. This knowledge may facilitate the development of antibodies that would augment the usefulness of diagnostic PSA immunoassays, e.g., antibodies that are highly specific for PSA or the various serological forms of PSA. A current concern with PSA antibodies is cross-reactivity with other members of the kallikrein family, in particular, human glandular kallikrein (hK2), which has 80% amino acid sequence identity with PSA *(3, 16, 17)*.

The purpose of the present investigation was to produce and characterize new anti-PSA antibodies, and to examine the epitope configuration of the PSA molecule. We report here the development of 11 anti-PSA monoclonal antibodies and their characterization with respect to affinity, cross-reactivity with hK2, epitope specificity, and practicability for assay development. This characterization enabled the development of highly sensitive immunofluorometric assays that differentiate between the serological forms of PSA through the selective detection of the free form of PSA.

Materials and Methods

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES

Immunizations and fusion. Mice were immunized with PSA purified from human seminal plasma (kindly provided by Dr. Thomas Stamey, Department of Urology, Stanford University, Stanford, CA). The procedure outlined by Gefter et al. *(18)* was followed to fuse immune splenocytes with murine myeloma cells.

Screening for antibodies specific for PSA. Hybridoma supernatants were screened using an ELISA to select antibodies showing specificity to PSA. Goat anti-mouse immunoglobulin (0.5μg in 100 μL of 50 mmol/L Tris buffer, pH 7.8) was immobilized onto polystyrene microtitration wells (Dynatech Laboratories, Inc.). This incubation was performed overnight at room temperature. The wells were washed six times with wash solution (150 mmol/L NaCl, 50 mmol/L Tris, 1 mmol/L NaN_3, 0.5 mL/L Tween 20), and the following series of incubations was performed at room temperature (volumes and amounts are per well): (*a*) 100 μL of hybridoma supernatant diluted 10-fold in a bovine serum albumin (BSA) diluent (50 mmol/L Tris, pH 7.8, containing 60 g/L BSA) for 30 min; (*b*) 100 μL of biotinylated PSA (0.1 μg) in 60 g/L BSA for 30 min; and (*c*) 100 μL of alkaline phosphatase-labeled streptavidin (0.005 μg; Jackson Immunoresearch) in 60 g/L BSA for 15 min. Each well was washed six times between each incubation. Diflunisal phosphate (DFP; 10 mmol/L stock in 10 mmol/L NaOH, prepared in house), diluted 10-fold in substrate buffer (100 mmol/L Tris, pH 9.1, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 7.5 mmol/L NaN_3), was added to each reaction and incubated for 10 min. Developing solution (1 mol/L Tris, 400 mmol/L NaOH, 3 mmol/L EDTA, 2 mmol/L Tb^{3+}) was then added to the DFP solution, and the resulting fluorescence was measured on a Cyberfluor 615 Immunoanalyzer (Nordion International). These time-resolved immunofluorometric procedures are discussed in more detail elsewhere *(19, 20)*.

Screening for antibodies specific for free PSA or PSA-ACT. Purified PSA-ACT was a gift from Dr. Thomas Stamey. Hybridoma supernatants containing PSA-specific antibodies were screened further to distinguish those specific for the free form of PSA from those that recognize both free PSA and PSA-ACT. Microtitration wells were coated with goat anti-mouse immunoglobulin as described above and washed, and the following series of incubations was performed at room temperature (volumes and amounts are per well): (*a*) 100 μL of hybridoma supernatant diluted 1:10 in 60 g/L BSA for 30 min; (*b*) 100 μL of free PSA (1 μg) or PSA-ACT (3 μg) in 60 g/L BSA for 1 h; (*c*) 100 μL of biotinylated rabbit anti-PSA polyclonal antibody (0.25 μg; Medix Biotech Inc.), which recognizes both captured free PSA or PSA-ACT, in assay buffer (50 mmol/L Tris, pH 7.8, 100 mL/L goat serum, 60 g/L BSA, 50 mL/L mouse serum, 10 g/L bovine γ-globulin, 5 mL/L Tween 20, 500 mmol/L KCl) for 1 h; and (*d*) alkaline phosphatase-labeled streptavidin as described previously. DFP and developing solution were added, and the fluorescence was measured.

Ascites production. Ascites fluid containing the selected antibodies was obtained from BALB/c mice that had been injected intraperitoneally with cloned hybridoma cells. Mice were initially injected with pristane to prepare the peritoneal cavity for tumor growth.

Antibody purification. Antibodies were purified from hybridoma culture supernatant and ascites fluid by affinity chromatography using the Affi-Gel Protein A MAPS II

system (Bio-Rad Laboratories) according to the manufacturer's specifications.

Antibody isotyping. The immunoglobulin subclass was determined using isotyping reagents from Sigma Immunochemicals, according to the manufacturer's instructions.

Determination of affinity constants. The affinity of each antibody was determined using the BIAcore 1000 system, which is based on surface plasmon resonance (SPR; Biacore AB). This system consists of a light source that provides incident light, a detection unit that measures reflected light, and a gold-coated sensor chip. SPR occurs when energy from the incident light reacts with free electrons on the gold film, thereby decreasing the amount of reflected light. The SPR signal is visualized as a dip in the intensity of the reflected light. Briefly, this technique involved immobilizing the antibody onto the surface of the sensor chip with a rabbit anti-mouse polyclonal antibody. PSA was injected across the surface of the sensor chip. Antigen-antibody binding caused a change in the refractive index at the surface of the sensor chip, thus altering the SPR signal. The BIAcore system is discussed in more detail elsewhere *(21)*.

Cross-reactivity of individual antibodies with hK2. The cross-reactivity of each PSA antibody with hK2 was assessed using Western blotting. All necessary equipment for Western blot analysis was obtained from Novex. Recombinant hK2 [a gift from Dr. Robert Wolfert at Hybritech Inc., San Diego, CA; described in Ref. *(22)*] was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions on 4–12% Tris-glycine polyacrylamide gels. Separated proteins were transferred electrophoretically to nitrocellulose membranes. After the membranes were blocked overnight at 4 °C with 50 g/L nonfat dry milk in 20 mmol/L Tris-HCl, 137 mmol/L NaCl, 1 mL/L Tween 20, pH 7.6, the membranes were cut into strips, and each strip was incubated with one PSA monoclonal antibody, followed by horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Corp.). Probing with an antibody specific for hK2 served as a positive control. PSA was electrophoresed in a separate well and was probed with a PSA-specific antibody. Biotinylated molecular weight markers were visualized by simultaneous reaction with a streptavidin-horseradish peroxidase and the secondary antibody. The blots were incubated for 1 min with enhanced chemiluminescence reagents (ECL; Amersham Corp.), as specified by the manufacturer, and exposed to x-ray film for detection of immunoreactive protein bands.

ANTIBODY PAIRING

Pairing experiments were performed using 11 antibodies generated in this study, in conjunction with 4 commercially available antibodies, coded 8301 and 8311 (Diagnostic Systems Laboratories) and F1 and F2 (BiosPacific). A pairing study was performed to determine whether the 15 antibodies could be paired with a set of reference anti-PSA antibodies included in the International Society for Oncodevelopmental Biology and Medicine (ISOBM) workshop (see below). Two additional pairing studies were performed to determine whether the 15 test antibodies could be paired with each other, either in the presence of immobilized PSA or in sandwich configurations. These experiments led to the generation of a PSA antigenic map and the development of immunoassays specific for free PSA. The inability to pair, i.e., to bind simultaneously to PSA, would indicate identical (no fluorescence detected) or overlapping (low fluorescence detected) epitopes.

Pairing with ISOBM workshop antibodies. Pairing studies were performed to compare the epitope specificity of the 11 antibodies generated here and 4 commercial antibodies with that of 12 reference anti-PSA monoclonal antibodies included in the ISOBM workshop as described elsewhere *(23)*. The reference antibodies belong to six major groups, and each group has a number of subgroups. One microgram of each of the 15 antibodies was incubated with 0.005 μg of purified PSA for 1 h. Fifty nanograms of 1 of the 14 reference antibodies, which had been labeled with europium (Eu^{3+}), was then applied for 1 h. This mixture was added to microtitration wells that had been coated with a PSA polyclonal antibody (Dako Diagnostics Canada Inc.) and incubated for 30 min. After the wells were washed, enhancement solution was added, and the fluorescence was measured. If the test antibody shared epitope specificity with the ISOBM workshop reference antibody, the labeled reference antibody would be unable to bind and no signal would be generated.

Antibody pairing by competitive binding to immobilized PSA. Additional investigation into epitope recognition by the 11 in-house antibodies and the 4 commercial antibodies was facilitated by competitive binding to immobilized PSA. PSA antigen was immobilized on microtitration wells, and the ability of any one antibody to block the binding of another was determined. The ability to block binding would indicate identical or overlapping epitope specificity. Polystyrene microtitration plates were coated with free PSA (0.5 μg in 100 μL of coating buffer per well) and incubated at room temperature overnight. After the wells were washed, they were incubated for 1 h with one of the 15 antibodies diluted in 60 g/L BSA (1 μg of antibody in 100 μL of BSA solution). The biotinylated form of one of the antibodies (0.25 μg in 50 μL of 60 g/L BSA) was then applied to each well and incubated for an additional 30 min. After the wells were washed, alkaline phosphatase-labeled streptavidin was applied as described above. DFP and developing solution were added, and the fluorescence was measured.

Antibody pairing in sandwich immunoassays. Sandwich immunoassays were performed with all possible combinations of the 11 in-house and 4 commercial antibodies to determine which pairing combinations enabled specific recognition of free PSA. In addition, this analysis confirmed the findings from the previous experiment in terms of epitope sharing or overlap. Microtitration wells were coated with each antibody (0.2 μg in 100 μL) as described above. The wells were washed after each of the following incubations at room temperature: (*a*) 100 μL of 0.5 and 5 μg/L free PSA diluted in a 60 g/L BSA diluent or 100 μL of 1.5 and 15 μg/L PSA-ACT for 1 h; (*b*) 100 μL of biotinylated antibody (0.05 μg in assay buffer) for 1 h; and (*c*) alkaline phosphatase-labeled streptavidin as described above. DFP and developing solution were added as described above.

free psa immunoassays

The above pairing experiments allowed us to identify two pairs of antibodies that were suitable for the selective detection of free PSA: antibody 9 for coating and antibody 2 for detection (designated 9/2); and antibody F2 for coating and antibody F1 for detection (designated F2/F1).

Cross-reactivity of free PSA immunoassays with PSA-ACT. Positive cross-reactivity was determined by incubation of various concentrations of PSA-ACT with the free PSA sandwich configurations. Microtitration wells were coated with 0.2 μg of antibodies 9 or F2 as described above. The wells were washed after each of the following series of incubations at room temperature: (*a*) 100 μL of PSA-ACT diluted in 60 g/L BSA to the following concentrations: 0, 0.15, 0.2, 2, and 10 μg/L; (*b*) 100 μL of biotinylated antibody (0.25 μg in assay buffer) for 1 h; and (*c*) alkaline phosphatase-labeled streptavidin as described above. DFP and developing solution were added as described above.

Negative cross-reactivity was determined by incubating free PSA and PSA-ACT simultaneously. Microtitration wells were coated with 100 μL of each antibody (0.2 μg) as described. The wells were washed as described after each of the following series of incubations at room temperature: (*a*) PSA-ACT (diluted in 60 g/L BSA at concentrations of 0, 0.03, 0.15, 0.5, 2, and 10 μg/L) in the presence of 0, 0.01, or 0.05 μg/L free PSA (giving a total volume of 100 μL) for 1 h; (*b*) 100 μL of biotinylated antibody (0.25 μg in assay buffer) for 1 h; (*c*) alkaline phosphatase-labeled streptavidin as described above. DFP and developing solution were added as described above.

Cross-reactivity was calculated by dividing the concentration of PSA-ACT detected by the free PSA immunoassay by the actual PSA-ACT concentration.

Cross-reactivity of free PSA immunoassays with hK2. The free PSA assays developed were tested for cross-reactivity with hK2. Microtitration wells were coated with 0.2 μg of antibodies 9 or F2 as described above. The wells were washed after each of the following series of incubations at room temperature: (*a*) 100 μL of hK2 diluted in 60 g/L BSA at the following concentrations: 0, 0.01, 0.1, 1, 10, and 100 μg/L; (*b*) 100 μL of biotinylated antibody (0.25 μg in assay buffer) for 1 h; and (*c*) alkaline-phosphatase-labeled streptavidin as described above. DFP and developing solution were added as described above.

Clinical samples. To determine the accuracy of the free PSA measurements obtained using the immunoassays generated in our laboratory, we compared values with those obtained with the Immulite Free PSA Kit (Diagnostic Products Corp.).

Fifty male serum samples were collected from the Department of Pathology and Laboratory Medicine at Mount Sinai Hospital in Toronto, Canada. Polystyrene microtitration wells were coated with 0.5 μg of antibodies 9 or F2 as described above. The wells were washed as described above after being coated and after each of the following series of incubations: (*a*) 20 μL of serum + 50 μL of assay buffer for 2 h; (*b*) 100 μL of biotinylated antibody (0.25 μg in assay buffer) for 30 min; and (*c*) alkaline phosphatase-labeled streptavidin as described above. DFP and developing solution were applied as described above. The concentrations of the free PSA preparations used for calibration were 0, 0.05, 0.2, 1, 5, and 20 μg/L. All calibrators and samples were analyzed in duplicate. Linear regression and Pearson correlation analyses were performed to compare PSA measurements by different assays.

Results

characterization of psa monoclonal antibodies

Recognition of free or total PSA. Of the 11 antibodies generated in this study, 10 recognized both free PSA and PSA-ACT, and thus were specific for total PSA; whereas 1 antibody (antibody 9) recognized only free PSA. Three of the commercial antibodies (8301, 8311, and F2) recognized total PSA, whereas F1 detected only free PSA. Immunohistochemistry demonstrated that all antibodies generated showed intense positive immunostaining of healthy prostatic ductal epithelium (data not shown).

Antibody isotyping. The absorbance at 450 nm was highest when goat anti-mouse IgG1 was used as the capture antibody. Therefore, all of the PSA monoclonal antibodies produced are of the IgG1 subclass.

Affinity constants. The affinity constant (K_a) and K_d (K_a^{-1}) for each antibody is listed in Table 1. K_a values ranged from 1.1×10^8 to 2.5×10^{10} L/mol. The antibody with the highest K_a was 8301. The K_a for antibody 8 was not measurable using first-order kinetics.

Cross-reactivity of antibodies with hK2. Antibodies 4, 5, 11, 8311, and F2 recognized hK2 electrophoresed under de-

Table 1. Affinity constants for PSA monoclonal antibodies.

Antibody	Free or total PSA	K_a[a]	K_d[b]
1	Total	8.8×10^{8}	1.1×10^{-9}
2	Total	3.4×10^{8}	2.9×10^{-9}
3	Total	9.6×10^{8}	1.0×10^{-9}
4	Total	1.8×10^{10}	5.5×10^{-11}
5	Total	4.5×10^{9}	2.2×10^{-10}
6	Total	1.2×10^{8}	8.4×10^{-9}
7	Total	4.6×10^{8}	2.2×10^{-9}
8	Total	NM[c]	NM
9	Free	1.2×10^{9}	8.2×10^{-10}
10	Total	1.1×10^{8}	8.7×10^{-9}
11	Total	2.1×10^{9}	4.9×10^{-10}
8301[d]	Total	2.5×10^{10}	4.0×10^{-10}
8311[d]	Total	6.3×10^{9}	1.6×10^{-10}
F1[d]	Free	1.3×10^{10}	7.9×10^{-11}
F2[d]	Total	1.0×10^{9}	10.0×10^{-10}

[a] L/mol.
[b] mol/L.
[c] NM, not measurable (see *Discussion*).
[d] The commercial antibodies used are coded 8301, 8311, F1, and F2.

naturing conditions (Fig. 1). PSA loaded in an adjacent well was probed with a commercial PSA antibody (8301) as a positive control.

ANTIBODY PAIRING AND EPITOPE MAP

Pairing studies were performed with the 11 in-house antibodies, in conjunction with the 4 commercial antibodies, to generate a PSA epitope map and to develop a sandwich-type immunoassay for free PSA. The fluorescence generated with each antibody pair is shown in Fig. 2. Two antibodies with identical or overlapping epitopes were unable to bind to the same PSA molecule simultaneously, thus generating a very low fluorescent signal, as shown by the filled circles. Antibodies specific for antigenic sites that do not overlap are able to "sandwich" the PSA molecule and generate a high fluorescent signal, represented by the open circles. Intermediate levels of fluorescence are shown as gray circles.

The ability of each of the 15 antibodies to inhibit binding of the 12 reference ISOBM workshop antibodies was also determined. This analysis enabled the antibodies

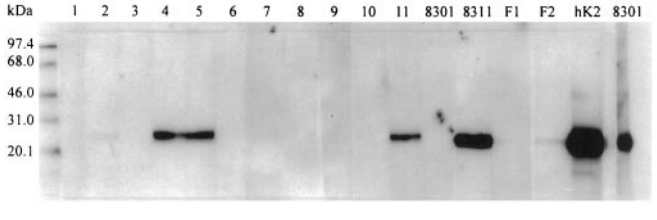

Fig. 1. Cross-reactivity of PSA antibodies with hK2.

Recombinant hK2 was electrophoresed on a 4–12% sodium dodecyl sulfate-polyacrylamide gel under nonreducing conditions. Probing was performed using 11 experimental (*antibodies 1–11*) and 4 commercial (*8301*, *8311*, *F1*, and *F2*) PSA monoclonal antibodies. PSA was run in a separate well and probed with the PSA commercial antibody coded 8301 (*PSA + 8301*).

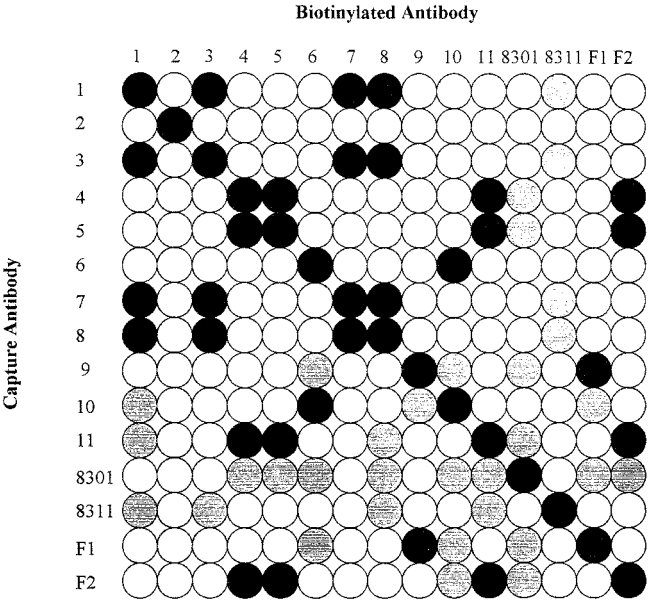

Fig. 2. Signal generated on antibody pairing.

Circles represent the relative levels of fluorescence produced upon combining each possible pair of antibodies in a sandwich configuration. (○), high fluorescence, >10 000 arbitrary units; (●), moderate fluorescence, 5000–10 000 arbitrary units; (●), low fluorescence, <5000 arbitrary units).

to be sub-classed into various groups, as described by Paus et al. *(23)* (Fig. 3). Group 1 represents antibodies binding to free PSA only, whereas groups 2–6 represents antibodies binding to free PSA and PSA-ACT. For group assignment, antibodies should inhibit 80% of the PSA-binding to, and should be unable to pair with, one specific ISOBM workshop antibody. Group 7 contains antibodies

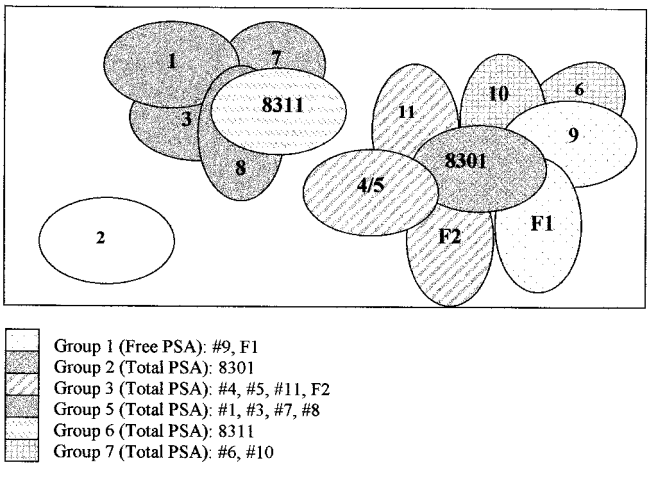

Fig. 3. Classes of PSA-specific antibodies according to ISOBM workshop epitope specificity and proposed epitope map for PSA.

Eleven in-house and 4 commercial antibodies were sub-classed on the basis of their ability to pair with 14 reference ISOBM PSA antibodies [described by Paus et al. *(23)*]. A map of the three antigenic domains was derived from the results of the competitive binding and pairing experiments. Antibodies *F1*, *F2*, *8301*, and *8311* are commercial antibodies. *Numbers* correspond to antibodies that bind to proposed epitopes. *Overlapping circles* indicate interfering sandwich formation. *Separated circles* indicate independent epitopes. No antibody was identified that belongs to group 4. *Antibody 2* appears to be distinct from all ISOBM and commercial antibodies.

that did not inhibit the binding of any of the ISOBM workshop antibodies.

When the 15 test antibodies were paired with each other in all possible combinations in the presence of immobilized PSA or in a sandwich configuration, the above results were confirmed and the positions of the epitopes relative to each other were determined. This analysis allowed us to generate a putative epitope map of PSA, shown in Fig. 3. Antibodies from group 5 (antibodies 1, 3, 7, and 8) were unable to pair with 8311 (group 6); therefore, these antibodies recognize a cluster of two closely spaced domains. Antibody 8301 (group 2) could not be paired with antibodies in groups 1 (antibodies 9 and F1), 3 (antibodies 4, 5, 11, and F2), or 7 (antibodies 6 and 10). Furthermore, antibodies in group 7 could not be paired with those in group 1. Therefore, a second antigenic domain appears to contain epitopes recognized by groups 1, 2, 3, and 7. Finally, the site recognized by antibody 2 appears to be unique because this antibody could be paired with any of the in-house or commercial antibodies.

FREE PSA IMMUNOASSAYS

The pairing studies allowed us to select two combinations of antibodies (9/2 and F2/F1) for use in free PSA immunoassays. The detection limit of each PSA assay was determined by the analysis of 20 replicates of the zero PSA calibrator. The detection limit of the assay, defined as the PSA concentration that corresponded to the fluorescence of the zero calibrator plus 2 SD, was determined to be 0.001 μg/L.

Cross-reactivity of free PSA immunoassays with PSA-ACT. Positive cross-reactivity of PSA-ACT was <0.5% in the free PSA immunoassays generated from in-house antibodies (9/2) and ≤1% for the immunoassay generated using the F2/F1 configuration. Negative cross-reactivity was determined by the simultaneous incubation of PSA-ACT and free PSA. Increasing concentrations of PSA-ACT did not interfere with the signal generated by free PSA for both free PSA assays, at ratios of PSA-ACT to free PSA up to 300.

Cross-reactivity of free PSA immunoassays with hK2. The cross-reactivity of both free PSA immunoassays (9/2 and F2/F1) with hK2 was determined. No signal was generated with hK2 at concentrations up 100 μg/L, indicating that this protein is not recognized by these sandwich configurations.

Clinical samples. To determine the accuracy of the PSA measurements obtained using PSA immunoassays generated with in-house antibodies and commercial antibodies, we compared male serum free PSA values with those obtained with the Immulite Free PSA Kit. Calibration curves were utilized to measure free PSA in male serum in immunoassays using the 9/2 and F2/F1 configurations.

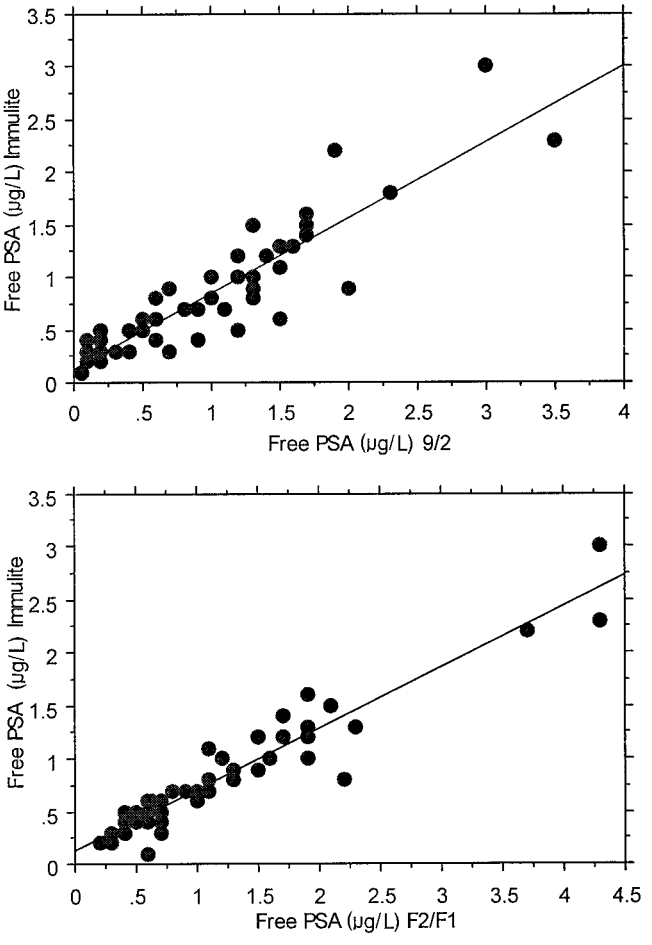

Fig. 4. Linear regression analysis of the results of free PSA measurements.

Comparison of free PSA in male sera measured with the Immulite Free PSA Kit and with a free PSA immunoassay developed with in-house antibodies (9/2; *top*) and one developed with commercial antibodies (F2/F1; *bottom*). The equations for the lines of regression are: for the assay with in-house antibodies (*top*), $y = 0.124 + 0.722x$, $r^2 = 0.801$; for the assay with commercial antibodies (*bottom*), $y = 0.134 + 0.577x$, $r^2 = 0.907$. All units are in μg/L.

The within-run and between-day imprecision (CV) was <10% for four male sera containing free PSA between 0.02 and 0.2 μg/L.

Linear regression and Pearson correlation analyses were performed to compare free PSA measurements by different assays (Fig. 4). The free PSA values obtained with the immunoassays using the 9/2 and F2/F1 combinations correlate fairly well with those obtained by the Immulite Kit, with r values of 0.90 and 0.95, respectively. The free PSA values obtained using the 9/2 or F2/F1 configuration, however, appeared to be consistently higher than those obtained using the Immulite Kit because the slopes of the linear regression plots were 0.6 and 0.7, respectively, and the y-intercepts were 0.13 (P = 0.002) and 0.12 (P = 0.05), respectively.

Discussion

Prostate cancer is the most commonly diagnosed cancer in North American males *(24)*. Over the past decade, PSA

has emerged as the most valuable marker in urologic oncology *(25)*. The utility of PSA as a tool for postsurgical monitoring, however, is considerably enhanced when ultrasensitive immunoassays (detection limit <0.1 μg/L) are used because conventional systems are not able to quantify the low concentrations of PSA in the sera of prostatectomized males *(19)*.

The potential of serum PSA measurements as a screening tool for prostate cancer remains controversial because of the lack of specificity of PSA for prostate cancer. Numerous nonmalignant conditions of the prostate, such as benign prostatic hyperplasia, are associated with increased PSA *(26)*. The ratio of free to total PSA has become an important variable for distinguishing between males with benign and malignant prostatic pathology. Shortly after the discovery of different molecular forms of PSA in serum *(8–10)*, it was demonstrated that PSA-ACT accounted for a higher fraction of serum PSA in patients with prostate cancer than in those with benign prostatic hyperplasia *(10)*. Although the total PSA concentration alone is neither sensitive nor specific enough for the early diagnosis of prostate cancer, the ratio of free to total PSA may improve both sensitivity and specificity *(10, 14)*. The development of highly sensitive immunofluorometric assays that differentiate between the different serological forms of PSA and those that selectively detect the free form of PSA would, therefore, have clinical relevance. Furthermore, ultrasensitive PSA analysis may have utility in breast cancer detection or diagnosis. It is now widely accepted that PSA is present in hormone-regulated female tissues such as the breast, and is detectable in female sera *(27)*. Recent studies have shown that the molecular forms of PSA in serum may differ between women with and without breast cancer *(28, 29)*, thus introducing an additional use for ultrasensitive immunoassays that discriminate between free PSA and PSA-ACT. Such studies could not be conducted with any commercially available free PSA assays because of insufficient sensitivity.

In the present study, 11 PSA monoclonal antibodies belonging to the IgG1 subclass were produced. The specificity of the antibodies for PSA was confirmed by immunohistochemical staining of healthy prostatic tissue. The affinity constants (K_a) of the antibodies produced ranged from 1.1×10^8 to 1.8×10^{10} L/mol. The affinity of antibody 8 could not be measured using first-order kinetics. It is likely that the binding of the first PSA molecule to the antibody inhibited the binding of a second molecule, leading to a two-step binding curve. The affinity could likely be predicted using a more complex model.

Pairing studies were performed to compare the epitope specificities of the antibodies produced with that of each other, with commercially available antibodies, and binding inhibition with ISOBM workshop antibodies. Three different antigenic clusters on the PSA molecule were distinguishable. One antigenic domain is formed by the epitope for which only antibody 2 is specific. The antigenic determinant of this antibody is unique, enabling it to be paired with any other PSA antibody. This epitope is specific for free PSA and is masked by the binding of PSA-ACT. The other two antigenic clusters are composed of a number of epitopes, as shown in Fig. 3. Antibodies F1 and 9, which belong to group 1 as described by Paus et al. *(23)*, preferentially bind free PSA. Two of the antibodies, 6 and 10, did not inhibit the binding of any of the ISOBM antibodies. This was probably because of their relatively low affinity, approximately 10^{-8} mol/L.

Pairing studies also led to the development of two novel ultrasensitive free PSA immunoassays that exhibit $<1\%$ cross-reactivity with PSA-ACT. Commercially available antibodies are used in one assay, whereas the other consists of antibodies generated in this study. Both immunoassays have a detection limit of 0.001 μg/L, which represents the lowest concentration of free PSA ever measured with an immunoassay. The functional sensitivity is usually two- to threefold higher, as described previously *(19)*.

We here present evidence that among the antibodies produced, those of groups 3 and 6 all appear to react with an epitope that is also present on the hK2 molecule. Importantly, it is also clear that a number of PSA monoclonal antibodies are very specific for PSA, with no cross-reactivity from hK2. PSA immunoassays designed to use antibodies such as these as the coating antibody should be free of hK2 interference.

The free PSA immunoassays were compared with the corresponding assay used for routine clinical PSA analysis, the Immulite Free PSA Kit. The two free PSA immunoassays correlated well with the Immulite Free PSA assay, but with somewhat lower slopes and significantly different *y*-intercepts. This is likely because of underestimation of free PSA by the Immulite assays in comparison with the in-house immunoassays, which have higher analytical sensitivity (0.05 vs 0.001 μg/L). The cross-reactivity with hK2 is not responsible for the discordance because neither of the free PSA assays produced detect this kallikrein.

In summary, we have produced 11 PSA-specific monoclonal antibodies that were used, in conjunction with 4 commercial PSA antibodies, to characterize the antigenic domains of the PSA molecule and to develop highly sensitive immunoassays for free PSA.

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