Equivalent Recognition of Free and ACT-Complexed PSA in a Monoclonal-Polyclonal Sandwich Assay is Conferred by Binding Specificity of the Monoclonal Antibody

Zeqi Zhou,¹ E. Glenn Armstrong,¹ Alexander Belenky,¹
James V. Freeman,² and Kwok K. Yeung^{1*}

¹Business Group Diagnostics, Immunodiagnostics Business Unit, Bayer Corporation, Tarrytown, New York

²Business Group Diagnostics, Immunodiagnostics Business Unit, Bayer Corporation, Elkhart, Indiana

The Bayer Immuno 1™ PSA Assay measures free and ACT-complexed PSA on an equimolar basis, although it uses a monoclonal antibody (MM1) for capture and polyclonal antibodies for detection. Competitive inhibition studies using antibodies directed at various epitopes on PSA and PSA-ACT demonstrated that the capture antibody, MM1, does not bind to free PSA simultaneously with antibodies against Epitope E which is exposed only in free PSA. Affinity studies showed that the affinity constants of MM1 for both free PSA and PSA-

ACT are similar. One explanation for the properties of MM1 is that it precludes the binding of antibodies to Epitope E due to steric hindrance. Alternatively, the binding of MM1 causes a conformation change within the free PSA molecule, so that Epitope E is altered in a way that causes a loss of binding affinity. The unusual properties of MM1 are responsible for the equimolar response of this monoclonal-polyclonal sandwich assay for free and ACT-complexed PSA. *J. Clin. Lab. Anal.* 12:242–249, 1998. © 1998 Wiley-Liss, Inc.

Key words: Bayer Immuno 1™ analyzer; PSA immunoassay; antibody binding specificity; equimolar response; Epitope E

INTRODUCTION

Prostate-specific antigen (PSA), a single chain glycoprotein of approximately 30 kilodaltons (kDa), is a member of the human kallikrein family of serine proteases (1, 2). Both chymotrypsin and trypsin-like activities have been reported (3–6). PSA is one of the major proteins found in seminal fluid (7). Originally, it was demonstrated to be specifically secreted by the epithelial cells of the prostate gland. Recently, Diamandis et al. identified PSA in female breast tissue and breast milk from female breast cancer patients (8, 9). Numerous studies have established this protein as an important, clinically useful biomarker for the management of patients with prostate cancer (10–13). The measurement of PSA in human sera has been demonstrated to be valuable in the early detection of prostate carcinoma. The American Cancer Society and the American Urological Society recommended that all men aged 50 and older be tested annually for prostate cancer using serum PSA in conjunction with digital rectal examination (14).

As a serine protease, PSA has been shown to form complexes with serine protease inhibitors such as α_1 -antichymotrypsin (ACT) and α_2 -macroglobulin (A2M) (15–22). Two laboratories discovered separately that PSA exists in serum in free form and in forms bound to various protease inhibitors (15, 20). Complexes of PSA with A2M are not measurable by currently employed immunoassays, and the

majority of immunoreactive PSA found in human serum is complexed with ACT (15). Stenman et al. (15) and Lilja et al. (20) demonstrated that serum from prostate cancer patients contains a higher proportion of ACT-complexed PSA than that of benign prostate hyperplasia (BPH) patients. Recent studies have suggested that the quantification of free PSA or PSA-ACT complexes and the determination of their ratios to total PSA in patient sera may assist in differentiating prostate cancer from BPH (15, 18, 19, 23).

A number of investigators have reported that commercial PSA assays may give different values for the same patient specimens (16, 22, 24). For example, the Yang Pros-Check® PSA assay yielded PSA values that were significantly higher than those from the Hybritech® Tandem®-R assay on the same samples (24). Another study demonstrated that the Ciba Corning ACS:180™ PSA assay and the Abbott IMx® PSA

Abbreviations: PSA, prostate specific antigen; ACT, alpha-1-antichymotrypsin; BPH, benign prostatic hyperplasia; BSA, bovine serum albumin; Immuno 1, Bayer Immuno 1™ analyzer; ALP, alkaline phosphatase; FITC, fluorescein isothiocyanate

*Correspondence to: Kwok K. Yeung, Ph. D., Business Group Diagnostics, Immunodiagnostics Business Unit, Bayer Corporation, Tarrytown, New York 10591-5097. E-mail: Kwok.Yeung.B@Bayer.com

Received 8 December 1997; Accepted 27 February 1998

assay yielded PSA values in serum samples from patients with prostate cancer and BPH approximately 2 to 3 times higher than the Hybritech Tandem-R PSA method (16). The reasons for these differences have been attributed chiefly to the calibrator compositions and differences in assay formats and antibodies (16, 25, 26). However, a recent report showed that although there is still variability among methods in reporting PSA values, the agreement is now better (23), presumably due to the availability of a PSA reference material and manufacturers of PSA assays having standardized the calibration curves to this reference (22).

In 1995, Stamey reviewed the effect of the immunoassay format on serum PSA quantitation (22). He noted that assays using a monoclonal-monoclonal antibody sandwich format generally yielded equimolar quantification of free and ACT-complexed PSA, whereas assays utilizing a monoclonal-polyclonal format did not. The latter assays tended to yield higher values for samples containing greater proportions of free PSA (22).

Use of a nonequimolar response assay to detect total PSA in patients suspected of having prostate cancer complicates interpretation of clinical results. This is because a distinct cut-off of 4 ng PSA/mL serum is used to differentiate those patients with a higher risk of prostate cancer from those who are likely to be normal or have benign diseases. In a nonequimolar method, results may be falsely above 4 ng/mL due to higher percentage of free/total PSA in normal patients and those with benign disease than those with prostate cancer. This is not the case in an equimolar method in which a patient sample with 4 ng/mL or less would be measured in a manner independent of the percentage of free/total PSA. Furthermore, the use of free/total PSA or complexed/total PSA ratios as a means of improving clinical specificity is compromised by the use of a nonequimolar total PSA method.

Accordingly, it is desirable to develop an assay with equimolar response to free and ACT-complexed PSA. In this article, we describe such a method which uses a monoclonal antibody and polyclonal antibody format, and discuss the immunological mechanism by which a monoclonal-polyclonal sandwich assay (the Bayer Immuno 1™ PSA assay) achieves an equimolar response to free and ACT-complexed PSA.

Antibodies directed at various epitopes of PSA were used. Previous epitope-mapping studies of free PSA and PSA-ACT using monoclonal antibodies revealed five or six major epitopes (26, 27), and five of the epitopes were named A through E (26). Monoclonal antibodies which bind to epitopes A, B, and C appear to react with both free and ACT-complexed PSA. Epitope D appears to be partially occluded by ACT (26, 27), and a monoclonal antibody to this epitope, 2E9, binds to free PSA at seven times higher affinity (27) than PSA-ACT. Epitope E appears to be a free PSA epitope and is completely occluded by PSA-complexation with ACT. Recently, Corey et al. conducted epitope-mapping studies using synthetic peptides and polyclonal antibodies against PSA (28). Their data

suggested that there are six major linear antigenic regions in PSA, consistent with results reported by Pettersson et al. (27).

MATERIALS AND METHODS

Antibodies and Conjugates

Alkaline phosphatase (ALP) and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO) and BioZyme (South Wales, UK), respectively. Antibodies used for the studies below include: 1) PSA19, PSA20, PSA30, and PSA66 (monoclonal antibodies) purchased from CanAg Diagnostics AB (Gothenburg, Sweden); the monoclonal antibody, M020105, purchased from Fitzgerald Industries International (Concord, MA); and monoclonal antibody ME2, from BiosPacific (Emeryville, CA). These antibodies were generated from mouse hybridoma clones by *in vitro* cultivation, and purified using Protein-A affinity chromatography. Their purity was determined by SDS-PAGE and the visible bands corresponded to IgG heavy and light chains under reducing conditions using the Pharmacia Phast System. Those antibodies were stored in a buffer containing 0.9% sodium chloride. PSA19, PSA20, PSA30, and ME2 specifically bind Epitope E of free PSA molecule, and PSA66 and M020105 bind both free and ACT-complexed PSA. 2) MM1 monoclonal antibody which specifically recognizes free PSA and PSA-ACT was produced in mouse ascites in-house (29). Conjugation of MM1 to fluorescein was performed according to the method of Hermanson (30). MP2 goat polyclonal antiserum was produced by immunization with free PSA purified from seminal plasma (Pyramid Biological Corp., Van Nuys, CA). Anti-PSA polyclonal antibodies were affinity-purified with PSA-Sepharose and then conjugated to ALP as previously described (29). A combination of MM1-FITC for capture and MP2-ALP for detection is utilized for the Bayer Immuno 1™ PSA assay. 3) The antibodies used as controls include rabbit antirecombinant glutathione S-transferase (GST, expressed in *E. coli*) purchased from Amard (Kew Victoria, Australia), which is a polyclonal antiserum purified from rabbit serum by Protein-A chromatography. Rabbit antihuman plasma ACT polyclonal antiserum was from BioDesign (Kennebunk, ME).

Antigens

Antigens used for those studies include free PSA from human seminal fluid with 98% purity, and PSA-ACT with 98% purity, both purchased from Scripps (San Diego, CA). Free PSA was stored in a buffer containing 10 mM Tris-HCl, 0.1% sodium azide, pH 8.0. PSA-ACT complex was stored in a buffer containing 10 mM sodium acetate, 150 mM sodium chloride, and 0.1% NaN₃, pH 5.6. Free PSA was conjugated with FITC by the method described by Hermanson (30) and FITC-PSA was used to conduct antibody competitive inhibition studies.

Buffers and Solutions

GS-5 buffer containing 100 mM Tris-HCl was prepared in-house and used for the reagent dilution. It contains 5% (heat-inactivated) normal goat serum which was purchased from Biocell Laboratories (Carson, CA). A 6% bovine serum albumin (BSA) solution was prepared with 25 mM Tris-HCl, 110 mM sodium chloride and 0.1% NaN₃, pH 8.0. It was used as the PSA calibrator base and for antigen dilutions. BSA was purchased from Sigma (St. Louis, MO).

The Bayer Immuno 1™ PSA Assay

The Bayer Immuno 1™ PSA assay is a commercially available sandwich assay which uses a monoclonal antibody for capture and purified polyclonal antibodies for detection of PSA. The monoclonal anti-PSA antibody (MM1) is conjugated to fluorescein (R1, 1.5 µg/mL) and the affinity-purified goat polyclonal antibodies are conjugated to ALP (R2, 6.5 µg/mL). Solutions of the two antibodies, at 65 µL/test, are incubated with specimen to form an immunocomplex (R1-PSA-R2) which is then captured by magnetic particles (10 µL/test) coated with anti fluorescein monoclonal antibodies. The particle bound immunocomplexes are washed and then 300 µL/test of 23 mM p-nitrophenyl phosphate are added and the rate of its conversion by bound ALP to p-nitrophenolate is measured. Calibration of the Bayer Immuno 1™ analyzer is performed using the Immuno 1 SET point® PSA calibrators, prepared from free PSA at concentrations of 0, 2, 10, 25, 50, and 100 ng/mL. A cubic-through-zero fitting algorithm is used to generate a standard calibration curve. Operation of the Immuno 1™ analyzer is performed according to the manufacturer's instructions (31).

Automated Immunoassay for Free PSA

The protocol used for the automated free PSA research assay was the same as that described above for the Bayer Immuno 1™ PSA assay except for the following changes: monoclonal antibody PSA30 specific for free PSA was conjugated to fluorescein (PSA30-FITC) to replace MM1-FITC for capture (R1), and combined with MP2-ALP to form a monoclonal-PSA-polyclonal antibody sandwich on the Bayer Immuno 1™ analyzer. The concentrations of antibodies in R1 (65 µL/test) and R2 (65 µL/test) were 10 µg/mL and 6.5 µg/mL, respectively. The sample volume was 55 µL/test and magnetic particle volume was 20 µL/test.

Specificity Studies of PSA30 and PSA66 Monoclonal Antibodies

The assay was conducted as described for the Bayer Immuno 1™ PSA assay except that PSA30-FITC (10 µg/mL) and PSA66-FITC (1.5 µg/mL) were used as R1. Free PSA or ACT-complexed PSA at concentrations of 0, 2, 4, 10, and 20 ng/mL were spiked into two SET point calibrators of the

Immuno 1 total PSA assay. These calibrators, 1 and 3, contained 0 and 10 ng/mL of free PSA, respectively, and were analyzed in the two assays.

Inhibition of Binding of PSA30-ALP to PSA-FITC by Various Antibodies

This assay was conducted as described for the Bayer Immuno 1™ PSA assay. PSA-FITC (0.5 µg/mL, 65 µL/test) was used as R1 and PSA30-ALP (0.2 µg/mL, 65 µL/test) used as R2 for detection. Various concentrations from 0 to 40 µg/mL of unlabeled antibodies were added to the Tris-HCl buffer containing 6% BSA for analysis. The following antibodies were examined for their abilities to inhibit binding of PSA30-ALP to PSA-FITC: PSA30, PSA66, MP2, MM1, and rabbit anti-GST.

Studies of MM1 Inhibition of PSA30 Binding to Epitope E

The assay was carried out as described for the Bayer Immuno 1™ PSA method except that MM1-FITC for capture as R1 was at 1 µg/mL (65 µL/test) and PSA30-ALP (4 µg/mL, 65 µL/test) was used for detection as R2. The Immuno 1 SET point free PSA calibrators (0-100 ng/mL, 20 µL/test) were used as samples for analysis. This experiment was designed to determine if MM1 monoclonal antibody competes with PSA30, a monoclonal antibody specific for free PSA Epitope E.

Studies of Equimolar Response Assays for Free and ACT-Complexed PSA using Different Antibody Combinations

The assays were conducted as described for the Bayer Immuno 1™ PSA method. In two of the methods, MM1-FITC was replaced with PSA66-FITC (1.5 µg/mL) and PSA30-FITC (10 µg/mL). Free PSA and PSA-ACT complex were made up in various proportions (100:0, 80:20, 50:50, 20:80, and 0:100) to give a total concentration of approximately 4 ng/mL. These PSA mixtures (20 µL/test) were analyzed as samples by the sandwich immunoassays.

Determination of Affinity Constants

The BIAcore System, CM5 Sensor Chip, and the Amine Coupling Kit containing N-hydroxy-succinimide (NHS), N-ethyl-N'-(3-diethyl-amino-propyl) carbodiimide (EDC), and ethanolamine hydrochloride were obtained from Pharmacia Biosensor (Piscataway, NJ). Immobilization of an affinity purified goat antimouse Fc specific "capture" antibody to the sensor chip was performed via primary amine groups according to the manufacturer's instructions. A continuous flow rate of 5 µL per minute was utilized. First, the surface of the sensor chip was conditioned with HBS buffer (pH 7.4) consisting of 10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, and

0.05% P20 surfactant. Following activation of the carboxymethyl dextran matrix of the sensor chip with 45 μ L of a 1:1 mixture of 0.1 M NHS and 0.4 M EDC, a goat antimouse Fc antibody (45 μ L, 25 μ g/mL) was immobilized to the sensor chip. Unreacted sites in the sensor chip matrix were inactivated by the addition of 45 μ L of 1 M solution of ethanolamine hydrochloride. Then, MM1 antibody was bound to the chip via the antimouse Fc antibody by addition of 5 μ L of MM1 antibody (5 μ g/mL) in HBS buffer. The concentration of antibodies was estimated by UV absorbance at 280 nm according to the formula 1 mg/mL = 1.4 absorbance units.

The antigen association and dissociation rate constants, and the corresponding association constants of MM1 for PSA and PSA-ACT, were determined using the BIAcore instrument. Binding analysis was performed using a sequential injection sequence at a continuous flow rate of 5 μ L per minute. Following binding of MM1 to the capture antibody on the CM5 chip, 5 μ L each of free PSA (100 or 500 μ g/mL) or PSA-ACT (25 μ g/mL) were added. Association and dissociation rate analysis were performed assuming a first order kinetics model ($A + B \leftrightarrow AB$) using BIA evaluation software, version 2.1. The surface of the sensor chip was then regenerated by the addition of 5 μ L of 100 mM HCl. Following removal of the HCl from the surface of the CM5 chip by rinsing with HBS, the chip was prepared for analysis of the next antigen by readdition of an antibody and then antigen. An antibody to deoxyripyridinolone was used as a negative control. Furthermore, PSA-ACT was used as a negative antigen control when a free PSA specific antibody ME2, was captured on the sensor chip.

RESULTS

Specificity of PSA30 and PSA66 Monoclonal Antibodies

Figure 1 shows that a sandwich immunoassay composed of PSA30-FITC as R1 and MP2-ALP as R2 gave a linear response with increased levels of free PSA. This phenomenon was true for two levels of calibrators (See Materials and Methods.). The combination of PSA30-FITC as R1 and MP2-ALP as R2, however, showed no increased response with increased PSA-ACT concentration. These data indicate that PSA30 binds to an epitope (Epitope E) that is hindered in the PSA-ACT complex and, therefore, is specific for free PSA. The small amount of detectable signal at elevated PSA-ACT concentration probably suggests that the PSA-ACT preparation is contaminated with free PSA, as indicated by the manufacturer (see Materials and Methods).

Inhibition of Binding of PSA30-ALP to PSA-FITC by Various Antibodies

As shown in Figure 2, PSA30, MP2, and MM1 all competed effectively for the binding of PSA30-ALP to PSA-FITC. Surprisingly, the slope of the binding inhibition curve using MM1 is steeper than that for PSA30, suggesting that MM1 blocks PSA30-ALP binding even more effectively than PSA30 itself. MP2, a goat polyclonal antibody, is also very effective in blocking PSA30-ALP binding to PSA-FITC, indicating that this polyclonal antiserum contains a species of antibody which binds to Epitope E on the PSA molecule. PSA66, which binds to PSA

Fig. 1. Specificity studies of PSA30 and PSA66 monoclonal antibodies. The assays were conducted as described in Materials and Methods. PSA30-FITC was paired with MP2-ALP in the sandwich assay on the Bayer Immuno

1TM system. The indicated concentrations of either free or ACT-complexed PSA were added to the calibrator levels 1 and 3 containing 0 and 10 ng/mL of free PSA, respectively.

Fig. 2. Inhibition of binding of PSA30-ALP to PSA-FITC by other antibodies. PSA-FITC and PSA30-ALP were used in a competitive binding assay as described in Materials and Methods. PSA-FITC was captured by

magnetic particles which were coated with antiluorescen antibodies. PSA30-ALP was used for detection. Five unlabeled antibodies at various levels were used to compete with PSA30-ALP for binding to PSA-FITC.

but is not specific for Epitope E, has insignificant inhibition. An irrelevant polyclonal antiserum, rabbit anti-GST, did not inhibit the binding of PSA30-ALP to PSA-FITC.

Monoclonal Antibody MM1 Inhibits Binding of PSA30 to Epitope E

No signal was detected at several levels of PSA added to a sandwich assay composed of MM1-FITC and PSA30-ALP antibody conjugates (data not shown) suggesting that no immunocomplex was formed. Two mechanisms may account for this finding. One is that the binding of MM1 changes the conformation of Epitope E on PSA so that PSA30 no longer recognizes it. Alternatively, the epitopes which MM1 and PSA30 bind may be in close proximity so that the binding of one antibody to PSA sterically inhibits the binding of the other.

Combination of MM1-FITC and MP2-ALP gives an Equimolar Response Assay for Free and ACT-Complexed PSA

MM1-FITC antibody conjugate in the Bayer Immuno 1TM PSA assay was replaced with either PSA66-FITC, PSA19-FITC, PSA20-FITC, or M020105-FITC conjugates for capture. All these methods were assayed with mixtures containing various proportions of free and ACT-complexed PSA. Results in Figure 3 show that the Bayer Immuno 1TM PSA assay has an equimolar response for free and ACT-complexed PSA despite its utilization of a polyclonal detection antiserum. In contrast, PSA66-FITC and, to a greater extent, M020105-

FITC, gave skewed responses, reflecting greater reactivity to free PSA than PSA-ACT complex in the method. These results indicate that, unlike MM1, monoclonal antibodies PSA66 and M020105 are ineffective in blocking the species of antibody in antiserum MP2 which bind to Epitope E on free PSA. Figure 3 also shows that the combinations of PSA19-FITC or PSA20-FITC which bind to Epitope E as R1, as well as MP2-ALP which was used as R2, are specific tests for free PSA.

Affinity Constants of MM1 for PSA and PSA-ACT

The affinity constants of MM1 for free PSA and PSA-ACT were determined using the BIAcore instrument as described in Materials and Methods. Association (k_a) and dissociation (k_d) rates were obtained from the sensograms after the addition of each antigen. Affinity constants of MM1, calculated by k_a/k_d for PSA and PSA-ACT were $1.9 \times 10^{11} \text{ M}^{-1}$ and $1.1 \times 10^{11} \text{ M}^{-1}$, respectively. For negative controls, an irrelevant antibody (specific for pyridinoline) was captured on the CM5 chip and then PSA was added, no increase in response unit (RU) was observed (data not shown). In addition, when a free PSA specific antibody, ME2, was captured on the chip, no observable RU was recorded upon addition of PSA-ACT. The binding of PSA to immobilized ME2 generated a sensogram from which the K_a was determined to be $2 \times 10^{10} \text{ M}^{-1}$ (data not shown).

DISCUSSION

The Bayer Immuno 1TM PSA method is based on a murine monoclonal (MM1) and a goat polyclonal antiserum (MP2)

Fig. 3. Combination of various antibodies and MP2-ALP yield equimolar response assay, skewed assay or free PSA specific assay. MM1-FITC was replaced with FITC labeled PSA66, M020105, PSA19, or PSA20 monoclonal antibodies for capture as R1 in the Bayer Immuno 1™ PSA assay. The goat polyclonal antibodies (MP2) conjugated to ALP (MP2-ALP) were

used for detection as R2 for all the assays. PSA19 and PSA20 are PSA Epitope E specific monoclonal antibodies. All these assays were conducted with five mixtures made up of various proportions of free PSA from 100% to 0% and ACT-complexed PSA from 0% to 100% in Tris-HCl buffer containing 6% BSA. Each mixture contains 5 ng/mL total PSA.

sandwich format. Compared to other PSA assays of similar design, the method would be expected to have a skewed response with free versus ACT-complexed PSA. Since no special precautions were taken in generating this polyclonal antiserum, one would expect a subpopulation of antibodies to Epitope E to be present in the polyclonal antiserum. These antibodies might be expected to result in a skewed response as demonstrated in other PSA assays which use a monoclonal-polyclonal sandwich format (16, 22, 32). However, as shown by previous studies with the Bayer Immuno 1™ PSA assay (33), this is not the case. The studies reported in this manuscript demonstrate that this is due to a unique property of the monoclonal capture antibody used in the assay.

PSA epitope-mapping studies with murine monoclonal antibodies have shown that there are five major, nonoverlapping epitopes, designated A through E, on a free PSA molecule. McCormack and his coworkers (26) reported that conformation-dependent as well as conformation-independent epitopes exist on PSA molecule. One of these epitopes, Epitope E, is completely masked when ACT binds to PSA. A very recent study indicates that six major antigenic regions are present in PSA as determined by linear epitope mapping and that certain monoclonal antibodies may inhibit PSA from complexation with ACT through peripheral occlusion of the antibody binding site, or through induction of conformational changes in PSA (28). Another epitope, Epitope D, is partially blocked or has a conformational change when ACT binds to PSA. This conclu-

sion was reached by studies with a monoclonal antibody that binds to free PSA with seven times higher affinity than that for PSA-ACT complex and also prevents binding of other antibodies to Epitope E (27). In contrast, three other epitopes (A, B, and C) are unaffected by the binding of ACT to PSA. These studies have provided the immunochemical basis for free PSA assays which use antibodies directed against Epitope E.

McCormack et al. (26) explained that the skewed response in nonequimolar response assays could be caused by either the length of incubation time or nature of the antibody reagents. For methods with short incubation times, the antigen and antibodies do not achieve equilibrium kinetics, causing a greater response to free PSA (M.W. of 30,000) than to PSA-ACT (M.W. of 90,000). Incubation time required for an equimolar response assay will vary depending on the assay format and antibody affinity constants for the two PSA forms. Another factor which contributes to the nonequimolar response of monoclonal-polyclonal sandwich PSA assays is the composition of the polyclonal antiserum. Polyclonal antiserum raised against free PSA will contain a subpopulation of antibodies directed to a region (Epitope E) which is obscured when PSA is bound to ACT. The binding of ACT to PSA shields this epitope and prevents the binding of the subpopulation of antibodies to the complex. Thus, fewer antibodies in the polyclonal antiserum bind to PSA-ACT, resulting in a relatively lower response in the immunological method than is achieved with an equivalent molar concentration of free PSA.

The Bayer Immuno 1™ PSA assay is novel in that it uses a monoclonal capture antibody and polyclonal detection antibodies and still generates an equimolar response to free and complexed PSA. Our studies have shown that this unexpected finding is not due to the composition of the polyclonal antibody (MP2), since it does contain antibodies that bind to Epitope E. On the contrary, the equimolar response for the assay is dependent on the properties of the monoclonal antibody (MM1) employed in the assay. Likely immunochemical mechanisms by which MM1 might act are blocking antibody binding to Epitope E through steric hindrance or by induction of a conformational change of PSA. A previous report (27) describes an antibody (2E9) which binds to Epitope D and completely inhibits the binding of antibodies to Epitope E. However, 2E9 binds with significantly greater affinity to free PSA than PSA-ACT complex, whereas, MM1 has similar affinity for free PSA and PSA-ACT complex. Another line of evidence which indicates that 2E9 and MM1 bind to different epitopes is that 2E9 binds to a linear sequence whereas MM1 binds to a conformation dependent epitope (34). The unique properties of MM1 together with its high affinity for both PSA and PSA-ACT appear important in the development of an equimolar assay.

The importance of PSA assays in the detection of prostate cancer demands the use of methods that are unaffected by the proportion of free and complexed forms in sera. Use of total PSA assays that do not have equivalent recognition to free and complexed PSA forms in patient serum samples can result in falsely elevated results and unnecessary biopsies. PSA sandwich assays that have been demonstrated to have equimolar responses typically utilize a combination of two monoclonal antibodies. Our studies have shown that an equimolar response can be achieved in a monoclonal-polyclonal sandwich assay for PSA by use of an unusual monoclonal antibody which confers equivalent recognition of free PSA and PSA-ACT complex. This is significant in achieving increased clinical specificity in the diagnosis of prostate cancer compared to other currently employed total PSA immunoassays with a monoclonal-polyclonal antibody format.

REFERENCES

- Wang MC, Valenzuela LA, Murphy GP, Chu TM: Purification of a human prostate specific antigen. *Invest Urol (Berl)* 17:159–163, 1979.
- Watt WK, Lee PJ, Timkulu TM, Chan WP, Loo R: Human prostate-specific antigen: Structure and functional similarity with serine proteases. *Proc Natl Acad Sci U S A* 83:3166–3170, 1986.
- Akiyama K, Nakamura T, Iwanaga S, Hara M: The chymotrypsin-like activity of human prostate-specific antigen, r-seminoprotein. *FEBS Lett* 225:168–172, 1987.
- Christensson A, Laurel CB, Lilja H: Enzymatic activity of prostatic-specific antigen and its reactions with extracellular serine proteinase inhibitors. *Eur J Biochem* 194:755–763, 1990.
- Lilja H: A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. *J Clin Invest* 76:1899–1903, 1985.
- Takagama TK, Fujikawa K, Davie EW: Characterization of the precursor of prostate-specific antigen. *J Biol Chem* 272:21582–21588, 1997.
- Lilja H, Abrahamsson PA: Three predominant proteins secreted by the human prostate gland. *Prostate* 12:29–38, 1980.
- Diamandis EP, Yu H, Sutherland DJA: Detection of prostate specific antigen immunoreactivity in breast tumors. *Cancer Res* 54:6344–6347, 1994.
- Yu H, Giai M, Diamandis EP, et al.: Prostate specific antigen is a new favorable prognostic indicator for women with breast cancer. *Cancer Res* 55:2104–2110, 1995.
- Papsidero LD, Wang MC, Valenzuela LA, Murphy GP, Chu TM: A prostate antigen in sera of prostate cancer patients. *Cancer Res* 40:2428–2432, 1980.
- Lange PH: Prostate specific antigen in diagnosis and management of prostate cancer. *Supplement to Urology* 36(5 suppl):25–29, 1990.
- Oesterling JE: Prostate specific antigen: A critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. *J Urol* 145:907–923, 1991.
- Armbruster DA: Prostate-specific antigen: Biochemistry, analytical methods and clinical application. *Clin Chem* 39:181–195, 1993.
- American Cancer Society: *Cancer facts & figures-1995*. American Cancer Society, Inc., Atlanta, GA. Page 11.
- Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, Alfthan O: A complex between prostate-specific antigen and α -1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: Assay of the complex improves clinical sensitivity for cancer. *Cancer Res* 51:222–226, 1991.
- Zhou AM, Tewari PC, Bluestein BL, Caldwell, GW, Larsen FL: Multiple forms of prostate-specific antigen in serum: Differences in immunorecognition by monoclonal and polyclonal assays. *Clin Chem* 39:2483–2491, 1993.
- Leinonen J, Lovgren T, Vornanen T, Stenman UH: Double-label time-resolved immunofluorometric of prostate-specific antigen and its complex with α -1-antichymotrypsin. *Clin Chem* 39:2098–2103, 1993.
- Christensson A, Bjork T, Nilsson O, et al.: Serum prostate specific antigen complexed with α -1-antichymotrypsin as an indicator of prostate cancer. *J Urol* 150:100–105, 1993.
- Lilja H: Significance of different molecular forms of serum PSA: The free, noncomplexed forms of PSA versus that complexed to α -1-antichymotrypsin. *Urol Clin North Am* 20:681–686, 1993.
- Lilja H, Christensson A, Dahlen U, et al.: Prostate-specific antigen in human serum occurs predominantly in complex with alpha1-antichymotrypsin. *Clin Chem* 37:1618–1625, 1991.
- Bjork T, Hulkko S, Bjartell A, Santagnese AD, Abrahamsson P-A, Lilja H: Alpha1-antichymotrypsin production in PSA-producing cells is common in prostate cancer but rare in benign prostatic hyperplasia. *Urology* 43:427–434, 1994.
- Stamey TA: Second Stanford conferences on international standardization of prostate specific antigen immunoassays: September 1 and 2, 1994. *Urology* 45:173–184, 1995.
- Dowell B, Munoz J, Weigand R: Multi-assay comparison of AxSYM total PSA assay using serum samples and the Stanford 90:10 PSA standard. *Tumour Biol* 18:36, 1997.
- Wu JT: Assays for prostate specific antigen (PSA): Problems and possible solutions. *J Clin Lab Anal* 8:51–62, 1994.
- Graves HCB, Wehener N, Stamey TA: Comparison of a polyclonal and monoclonal immunoassay for PSA: Need for an international antigen standard. *J Urol* 144:1516–1522, 1990.
- McCormack RT, Rittenhouse HG, Finlay JA, et al.: Molecular forms of prostate-specific antigen and the human kallikrein gene family: A new era. *Urology* 45:729–744, 1995.
- Pettersson K, Piironen T, Seppala M, et al.: Free and complexed prostate-specific antigen (PSA): In vitro stability, epitope map, and development of immunofluorometric assays for specific and sensitive detection of free PSA and PSA- α -1-antichymotrypsin complex. *Clin Chem* 41:1480–1488, 1995.

28. Corey E, Wegner SK, Corey MJ, Vessella RL: Prostate-specific antigen: Characterization of epitopes by synthetic peptides mapping and inhibition studies. *Clin Chem* 43:575–584, 1997.
29. Zhou Z, Armstrong G, Yeung KK: Monoclonal antibodies that bind equally to free and complexed analyte in a sandwich immunoassay. *European Patent Application* EPO 789, 032.
30. Hermanson GT (ed.): *Bioconjugate Techniques*. Academic Press, San Diego, 1996.
31. Technicon Immuno 1[®]System Prostate Specific Antigen (PSA). *Bayer Diagnostics*. Publication No. DA4-1207A94, January:1996, Issued: 1996.
32. Blase AB, Sokoloff RL, Smith KM: Five PSA methods compared by assaying samples with defined PSA ratios. *Clin Chem* 43:483–485, 1997.
33. Zhou Z, Ng CP, Very DL, Allard WJ, Yeung KK: Technicon Immuno 1[®] PSA assay measures both free and α -1-antichymotrypsin-complexed prostate-specific antigen on an equimolar basis. *J Clin Lab Anal* 10:155–159, 1996.
34. Zhou Z, Barnett TR, Very DL, et al.: Expression of human recombinant prostate specific antigen and specificity analysis of a panel of fifty-three PSA-specific antibodies to native and recombinant PSA. *Tumour Biol*. In press.