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Novel immunoassay for the measurement of complexed prostate-specific antigen in serum

W. Jeffrey Allard,* Zeqi Zhou, and Kwok K. Yeung

Serum prostate-specific antigen (PSA) is an effective diagnostic tool for detection of prostate cancer (CaP) at an early and potentially curable stage, but specificity is low. Studies have shown that the proportion of serum PSA complexed with α-1-antichymotrypsin (ACT) is higher in men with CaP than in men with benign prostate disease. We developed a novel immunoassay for complexed PSA based on the unique binding properties of a monoclonal antibody that fails to bind free PSA in the presence of antibodies specific for free PSA. The assay measured mixtures of free and complexed PSA accurately, and the measured values of free + complexed PSA in artificial mixtures and in patient sera were equivalent to the measured value of total PSA. Both the serum concentration and the proportion of complexed PSA was substantially higher in patients with CaP compared with patients with benign prostate disease. The cPSA assay may have utility in improving specificity in screening for prostate cancer.

Prostate specific antigen (PSA)[1] is a neutral serine protease composed of a single polypeptide chain of 237 amino acids, and which has a mature M_r of ~33 000 *(1)*. Although PSA is synthesized in other tissues *(2, 3)*, substantial concentrations of PSA are found in serum, almost entirely as a result of synthesis by the prostatic epithelium. Studies from several laboratories have established that measurement of serum PSA has value in monitoring patients with diagnosed prostate cancer (CaP) and in the early detection of localized, and therefore potentially curable, CaP *(4–6)*. Despite the widespread acceptance and use of serum PSA for early detection of CaP, the specificity of PSA testing is relatively low. In fact, several recent studies have shown that the yield of cancer in a screening population is only 22–30% *(7–9)*, which means that 70–80% of all biopsies are performed on men who do not have cancer. As regular screening for CaP becomes the standard of care and as the clinically obvious, advanced cases of CaP are detected in the male population, improved specificity in the detection of CaP is needed to avoid costly and unnecessary biopsies.

Several approaches have been suggested to improve the specificity of PSA testing. These include the use of serum PSA and prostate gland volume to develop a ratio termed PSA density *(10)*, longitudinal measurement of serum PSA values to develop the rate of increase in PSA per year, or PSA velocity *(11)*, and the application of age-adjusted reference ranges to compensate for the known increase in prostate gland volume in men over the age of 50 years *(12)*. Despite promising evidence in the clinical literature, none of these approaches has gained widespread acceptance. Another option to improve PSA specificity stems from the observations of Stenman et al. *(13)* and Lilja et al. *(14)*, who noted that PSA in serum exists in several forms including free, uncomplexed PSA and PSA complexed to several protease inhibitors, including α-2-macroglobulin, α-1-antichymotrypsin, and α-1-antitrypsin. In addition, these groups demonstrated that the proportion of PSA complexed with ACT increases as a function of the total PSA concentration and that the majority of immunoreactive PSA in cancer patients is in complex with ACT *(13–15)*. Accurate measurement of complexed PSA has proven difficult, however, because of technical problems with two-site sandwich assays for PSA-ACT. This may be because of the high concentrations of free ACT found in serum or because of complexation of ACT with other proteases, such as cathepsin G, which may cross-react in assays designed to measure PSA-ACT *(16, 17)*. Several options have been proposed to overcome these problems, such as the addition of heparin *(16)*, the use of Super Block *(18)*, addition of antibodies to cathepsin G *(17, 19)*, and the use of antibodies selective for PSA-ACT with reduced binding to free PSA or ACT alone *(20)*. None of these approaches, however, has been found to eliminate the problems of over- and underrecovery of PSA-ACT complexes in se-

Bayer Corporation, Business Group Diagnostics, 511 Benedict Ave., Tarrytown, NY 10591.
*Author for correspondence. Fax 914-524-2195; e-mail jeffrey.allard.b@bayer.com.
[1] Nonstandard abbreviations: PSA, prostate-specific antigen; CaP, prostate cancer; and ACT, α-1-antichymotrypsin.
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rum. Because of these problems, subsequent investigations focused on the use of a ratio of free and total PSA. Numerous studies have now demonstrated that the addition of free PSA to the test panel of total PSA and digital rectal examination yields improved specificity for discrimination of patients with benign and malignant prostate disease *(19, 21, 22)*.

In the studies reported here, we explored a different approach to the measurement of complexed PSA. Previous results from our laboratory have shown that the Bayer Immuno 1™ PSA Assay uses a monoclonal antibody for capture that recognizes both free and complexed PSA, but which, when bound to free PSA, precludes the binding of other antibodies specific for the free form of PSA *(23)*. We exploited the properties of this antibody to develop a novel assay for the measurement of PSA in complex with protease inhibitors. We show here that this assay is accurate, and that both the absolute amount as well as the proportion of complexed PSA is higher in the serum of patients with CaP than in those without cancer.

Materials and Methods

MATERIALS

Anti-PSA antibodies used in these studies include PSA19, PSA20, and PSA 30, which are monoclonal antibodies purchased as purified immunoglobulin from CanAg Diagnostics AB and stored in a buffer containing 10 mmol/L sodium phosphate, pH 7.4, and 150 mmol/L NaCl. The monoclonal antibody ME2 was purchased from BiosPacific and stored in a buffer containing 15 mmol/L KPO_4, pH 7.4, 150 mmol/L NaCl, and 1 g/L sodium azide. Each of these antibodies specifically recognizes the free form of PSA and does not bind to complexed PSA. The purity of each of the antibody preparations was found to be at least 95%, as tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, using the Pharmacia Phastgel Electrophoresis System followed by quantitative densitometric scanning. Free PSA from human seminal fluid and PSA-ACT were obtained from Scripps Laboratories and shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 96% and 97–98% pure, respectively. Free PSA was stored in a buffer containing 10 mmol/L Tris, pH 8.0, and 1 g/L sodium azide; PSA-ACT was stored in a buffer containing 10 mmol/L sodium acetate, pH 5.6, 150 mmol/L sodium chloride, and 1 g/L sodium azide.

AUTOMATED IMMUNOASSAYS FOR TOTAL AND FREE PSA

The Bayer Immuno 1 PSA Assay (Bayer Diagnostics) is a commercially available sandwich immunoassay that uses a monoclonal antibody for capture (termed the MM1 monoclonal antibody in the studies reported here) and affinity purified goat polyclonal antibodies conjugated to alkaline phosphatase for detection. This assay, which has been described in detail previously, provides equimolar detection of free and complexed PSA, based on properties of the monoclonal antibody used for capture *(23, 24)*. The free PSA assay used in these studies used antibody PSA19, a free PSA-specific monoclonal antibody, for the capture phase. All other conditions were the same as those used in the total PSA assay; this free PSA assay format has been described in detail previously *(24)*.

PATIENT SAMPLES

Serum samples from 86 consenting patients with biopsy-confirmed CaP and from 103 patients with biopsy-confirmed benign prostate disease were purchased from Bioclinical Partners. Samples were drawn before the biopsy procedure and were stored at −80 °C for up to 4 years before use. Information on the stage and grade of CaP was not available for these samples. All samples were thawed and tested in each of the three immunoassays (total, free, and complexed PSA) in one run on the Immuno 1 system and during one freeze-thaw cycle.

Results

OPTIMIZATION OF AN IMMUNOASSAY FOR COMPLEXED PSA

Previous work in our laboratory established that the Bayer Immuno 1 PSA Assay quantifies free PSA and complexed PSA on an equimolar basis *(23, 24)*. We found that the basis of equimolar detection of free and complexed PSA in the Immuno 1 PSA Assay results from the unique binding properties of the MM1 antibody used for capture. Binding of the MM1 monoclonal antibody to free PSA precludes binding of both monoclonal and polyclonal antibodies that are specific for the free form of PSA *(23)*.

The epitope recognized by free PSA-specific antibodies has been termed the E epitope. If the capture antibody in the Immuno 1 PSA Assay inhibits the binding of anti-E-specific antibodies, then the binding of antibodies to the E epitope may inhibit binding of the MM1 antibody. To test this hypothesis, we added E epitope-specific monoclonal antibodies over a range of concentrations to Immuno 1 PSA Assay calibrators containing only free PSA at concentrations of 25 and 10 μg/L (25 and 10 ng/mL). After a 30-min incubation at room temperature, we measured the reactivity of free PSA in the Immuno 1 PSA Assay. Results shown in Fig. 1A demonstrate that antibodies PSA19, PSA20, and PSA30 did reduce the reactivity of free PSA at 25 μg/L (25 ng/mL) in the Immuno 1 total PSA Assay. None of these antibodies reached saturation in their inhibition of free PSA reactivity, even at 200 mg/L, which represents a 2000-fold molar excess of antibody to PSA. This suggested that antibodies PSA19, PSA20, and PSA30 may be of low affinity relative to that of MM1. In addition, maximum inhibition of free PSA reactivity was 90% using

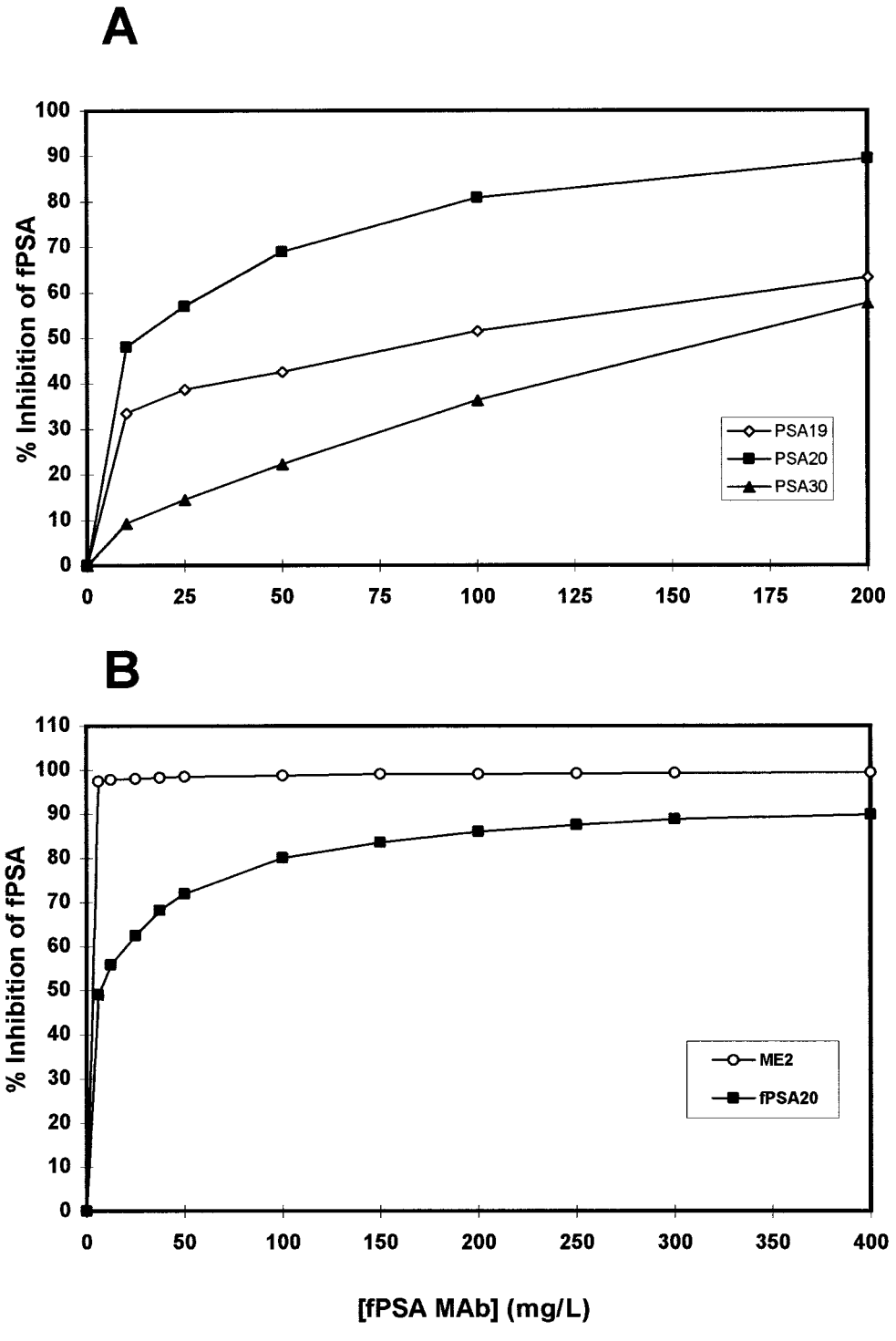

Fig. 1. Inhibition of immunoreactivity of free PSA in the total PSA assay.

Antibodies specific to free PSA were added over a range of concentrations to samples containing free PSA in buffer containing 60 g/L bovine albumin and tested in the Immuno 1 PSA Assay after a 30-min incubation. (*A*) Free PSA antibodies PSA19, PSA20, and PSA30 at 0–200 mg/L were added to Immuno 1 PSA Assay calibrator 4 with free PSA at 25 μg/L (25 ng/mL). (*B*) Free PSA antibodies PSA20 and ME2 at 0–400 mg/L were added to Immuno 1 PSA Assay calibrator 3 with free PSA at 10 μg/L (10 ng/mL).

these antibodies. Additional optimization showed that antibody PSA20 inhibited 93% of free PSA reactivity at 300 mg/L, but this concentration is too high for practical application to automated immunoassay formats. We therefore tested additional anti-E-specific monoclonal antibodies, and results with the free PSA-specific antibody ME2 are shown in Fig. 1B. Preincubation of free PSA at 10 μg/L (10 ng/mL) with the ME2 antibody gave virtually quantitative inhibition of free PSA reactivity in the Immuno 1 PSA Assay at antibody concentrations of <10 mg/L. These results formed the basis of an automated immunoassay specific for complexed PSA, shown diagrammatically in Fig. 2. An excess of ME2 antibody is used to reduce reactivity of free PSA in the Immuno 1 PSA Assay to <3%; therefore, >97% of the reactivity in the cPSA assay is because of complexed PSA.

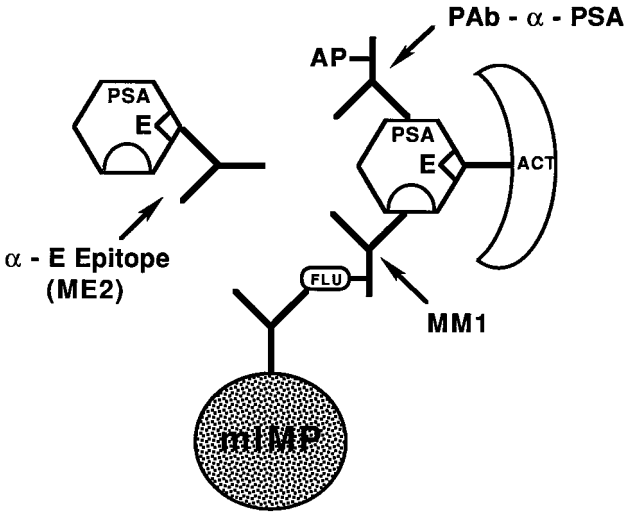

Fig. 2. Diagrammatic representation of the principle of the Immuno 1 cPSA assay.

AP, alkaline phosphatase used as an enzymatic tracer; *E*, the E epitope, which is the region on PSA that is sterically hindered by association of PSA with ACT; and *mIMP*, the magnetic particles coated with antibodies to fluorescein, which are used to capture the fluorescein-labeled MM1 antibody.

PERFORMANCE OF THE cPSA IMMUNOASSAY USING SUPPLEMENTED SAMPLES WITH VARYING PROPORTIONS OF FREE AND COMPLEXED PSA

In our initial efforts to optimize an immunoassay for complexed PSA, anti-E antibodies were preincubated with samples before measurement on the Immuno 1 instrument. In subsequent studies, we optimized the addition of anti-E antibody to provide a fully automated immunoassay format. In this format, the anti-E antibody is added directly into the compartment containing the alkaline phosphatase-labeled polyclonal anti-PSA used for the detection phase, to give a final concentration of anti-E antibody of 38 mg/L. We then prepared mixtures with varying proportions of free PSA and PSA-ACT at a total concentration of approximately 4 μg/L (4 ng/mL) PSA and tested these preparations in the Immuno 1 total, free, and complexed PSA assays. Results shown in Fig. 3 demonstrate that each of the PSA assays measured the mixtures of free and complexed PSA accurately. The Immuno 1 total PSA Assay demonstrated an equimolar response, as expected. The free PSA assay showed a decreasing response as the proportion of free PSA decreased, whereas the complexed PSA assay gave increasing values. When the values obtained with the free and complexed PSA assays were added and compared with concentrations measured by the total PSA assay, the sums of the free PSA and complexed PSA values were within 5% of the measured concentrations of total PSA.

PRELIMINARY MEASUREMENT OF COMPLEXED PSA IN PATIENTS WITH BENIGN AND MALIGNANT CaP

We tested the clinical utility of the cPSA assay, using sera from 86 patients with biopsy confirmed CaP and from 103 patients with biopsy confirmed benign prostate disease. Each serum sample was tested on the Immuno 1 analyzer with the total, free, and complexed PSA assays. Results shown in Table 1 demonstrate that the mean complexed

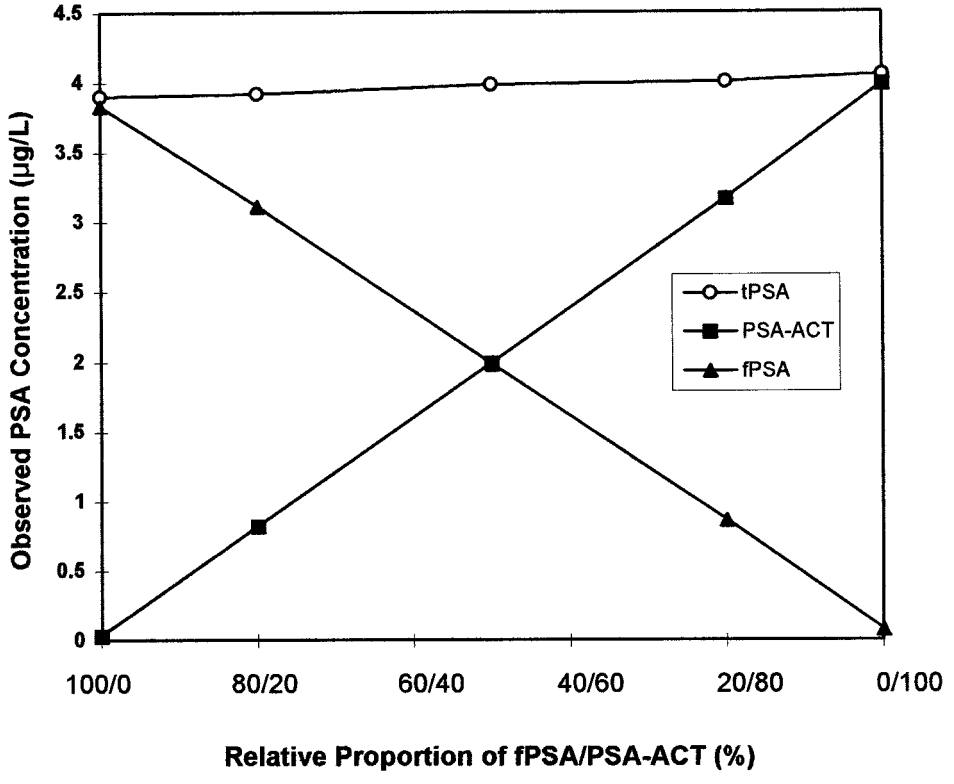

Fig. 3. Recognition of PSA forms by assays for total, complexed, and free PSA.

PSA and PSA-ACT were mixed at varying proportions at a total concentration of 4 μg/L (4 ng/mL) and tested in the Immuno 1 assays for total, free, and complexed PSA.

PSA concentration in patients with CaP was higher than in patients without cancer, and the proportion of complexed PSA was also higher in men with CaP (95%) compared with men without cancer (87%). The mean values for free PSA + cPSA gave values that were 107% and 106% of the mean values for total PSA in the benign and malignant prostate disease groups, respectively. Very similar results were obtained using regression analysis for a comparison of measured free PSA + measured cPSA, compared with measured total PSA (data not shown).

We also estimated the Decision Limit for cPSA. To maintain current standards of sensitivity for CaP detection, cPSA must demonstrate a sensitivity at least equivalent with total PSA. Therefore, rather than develop a Decision Limit based on healthy individuals, we estimated the highest value of cPSA that would provide equal sensitivity for cancer detection to total PSA at the accepted cutoff value of 4.0 μg/L (4 ng/mL). The sensitivity of total PSA in the CaP population in this study was 89%. To achieve the same sensitivity, the Decision Limit for cPSA was determined to be 3.75 μg/L (3.75 ng/mL).

Relative proportion of complexed PSA in serum of patients with benign prostate disease and CaP

Previous studies have shown that the proportion of PSA complexed to ACT is higher in patients with CaP compared with those with benign prostate disease *(13)*. We examined the proportion of complexed PSA as a function of the total PSA concentration, and the results are shown in Fig. 4. Only a slight trend can be seen toward increasing proportions of complexed PSA with increasing concentrations of total PSA. The clustering of patients with CaP in the upper right portion of Fig. 4 suggests that cancer patients do in fact have increased cPSA values, but no positive trend in the proportion of complexed PSA with total PSA could be seen for the cancer or benign prostate disease patient groups.

Discussion

The MM1 monoclonal antibody has unique binding properties for the PSA molecule. This antibody recognizes both PSA in the free form and complexed to ACT, but it precludes the binding of antibodies specific to the free form of PSA, such as ME2 *(23)*. It is unlikely that this phenomenon is the product of simple steric hindrance. MM1 binds both free and complexed PSA with approximately equal affinity and, therefore, does not recognize an epitope that is sterically hindered by the association of PSA with ACT. If the MM1 and ME2 monoclonal antibodies bind to unique epitopes, then the cross-inhibition observed must be because of a conformational change in the PSA molecule as a consequence of antibody association. Interestingly, if the PSA molecule undergoes a change in conformation as a result of anti-E antibody binding, this conformational change must be different from that brought about by the association of PSA with ACT. This is because the MM1 antibody binds to PSA-ACT complexes but not to free PSA in association with anti-E antibodies. It is likely that the ME2 antibody inhibits the reactivity of free PSA better than other anti-E antibodies tested because of its very high intrinsic affinity ($K_a = 2 \times 10^{10}$ L/mol). An alternative possibility is that the PSA19, PSA20, and PSA30 antibodies may bind to PSA with high affinity, but bind only partially within the E epitope and therefore only partially inhibit MM1 reactivity.

The reactivity of the MM1 antibody with PSA is similar to that reported for the 2E9 antibody, which also reacts with free and complexed PSA but does not form sandwiches with antibodies to free PSA *(16)*. However, the 2E9 antibody differs from MM1 in two key characteristics: 2E9 binds to reduced and denatured PSA *(16)*, whereas the MM1 antibody does not *(25)*; and 2E9 has a lower affinity for PSA-ACT than for free PSA, whereas the MM1 antibody binds to both forms with similar very high affinity, $K_a = 2 \times 10^{11}$ L/mol *(23)*.

The cross-inhibition of the MM1 monoclonal antibody with other antibodies to the E epitope of PSA forms the basis of a novel immunoassay format for the accurate measurement of PSA in complex with protease inhibitors. The assay described here is identical to the current Bayer Immuno 1 PSA Assay, which measures total PSA, except that the ME2 antibody is added in large molar excess. It is not clear at present which complexes of PSA with protease inhibitors react in the cPSA assay. Preliminary experiments have shown that the predominant reactivity is with PSA complexed to ACT (data not shown), but we cannot rule out reactivity with PSA complexed to other inhibitors, such as α-1-antitrypsin, protein C inhibitor, or inter-alpha-trypsin inhibitor. An additional possibility is that, if MM1 and ME2 bind to different epitopes on free PSA, then some forms of free PSA that are not recognized by ME2 could be bound by MM1. We consider this unlikely because the results shown in Table 1 suggest that the addition of measured free and complexed PSA is approximately equal to the average value for the measurement of total PSA.

Table 1. Summary of results for free, total, and complexed PSA values in patients with benign and malignant prostate disease.

Disease group	n	fPSA[a]	Mean values, cPSA	tPSA	f+c/t[b]
Benign	103	0.85 (1.01)	3.89 (5.48)	4.46 (5.90)	1.06
Cancer	86	1.41 (2.19)	10.59 (14.80)	11.16 (15.15)	1.07

[a] All measurements of PSA are in μg/L. Values in parentheses are standard deviations.
[b] Ratio of free + complexed PSA to total PSA.

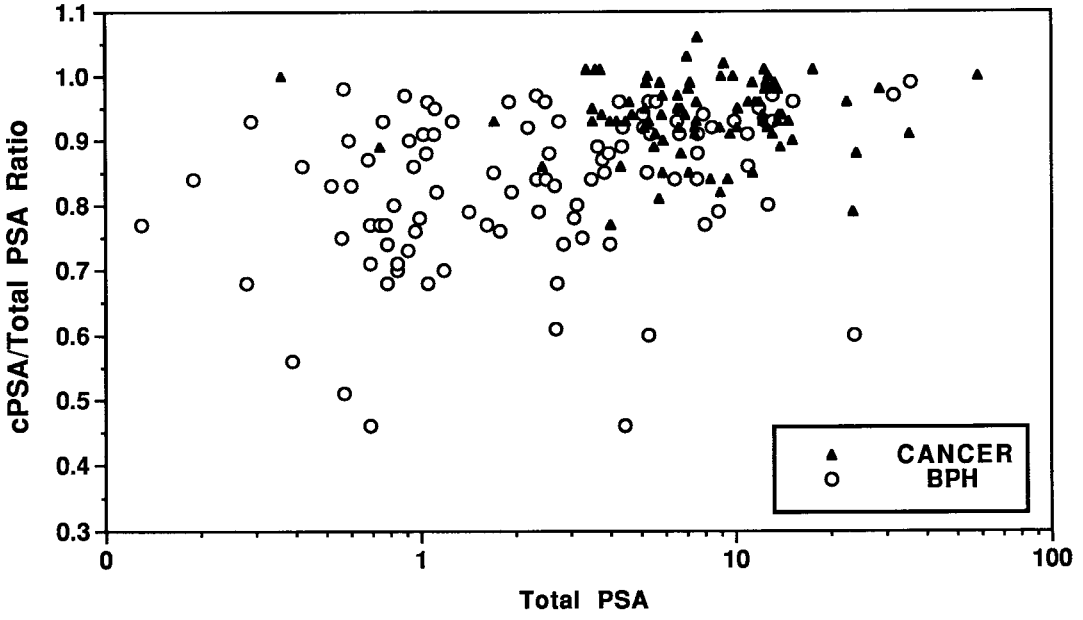

Fig. 4. Relationship between the total PSA concentration (note logarithmic scale) and the proportion of complexed PSA in sera from patients with and without CaP.

A total of 189 patients with either biopsy confirmed CaP (n = 86) or benign prostate disease (n = 103) were assayed using the Immuno 1 total and complexed PSA assays (1 μg/L = 1 ng/mL).

We have shown that the measurement of complexed PSA using the present assay is accurate in two ways. First, when PSA and PSA-ACT were mixed in vitro and measured using assays for total, free, and complexed PSA, the measured concentration of free + complexed PSA was equal to the measured concentration of total PSA for each of the mixtures (Fig. 3). In addition, for the serum samples from patients with and without cancer, the measured values for free + complexed PSA were approximately equal to the measured values for total PSA (Table 1). It is unlikely that the small overrecovery of free + complexed PSA compared with total PSA is the result of false high measurements using the total PSA assay, because this assay provides PSA concentrations that agree almost exactly with PSA measured by biophysical means *(24)*, and the same is true for the cPSA assay (data not shown). The free PSA assay used in these studies is a research assay that has been partially characterized and may give false high values. In any event, the discrepancy is slight, and taken together, these data demonstrate that the cPSA assay measures complexed PSA accurately in vitro and in vivo.

Previous attempts to measure PSA-ACT complexes used antibodies to PSA coated onto a microplate or beads for capture, and labeled anti-ACT for the detection phase *(13, 14, 17, 18, 26)*. These formats have been plagued with falsely increased values because of nonspecific binding of cross-reactive molecules. Several approaches have been used to combat this problem, such as the addition of heparin *(16)*, antibodies to cathepsin G with uncoated latex particles *(17)*, and Super Block *(18)*. These approaches have met with limited success. The addition of an antibody specific for free PSA to the Immuno 1 total PSA Assay avoids the problems of nonspecific interference by rendering free PSA nonreactive in the immunoassay.

The cPSA assay format was used to compare the proportion of complexed PSA in patients with benign and malignant prostatic disease. As shown in Fig. 4, no direct relationship between the proportion of complexed PSA and total PSA values could be seen. Previous studies examined similar populations and found contradictory results. Stenman et al. *(13)* found that the fraction of complexed PSA increases roughly in proportion to total PSA concentrations in men with benign prostate disease and cancer. Subsequent studies failed to reproduce this correlation, and showed results similar to ours *(21, 27)*. In all cases, however, the proportion of PSA complexed with ACT has been found to be higher in men with CaP compared with those who do not have cancer. In addition, in the present study sera from all men with CaP were found to contain >77% complexed PSA. This is in contrast with sera from men with benign prostatic disease, where the proportion of complexed PSA ranged from 46% to 98%.

The use of the cPSA immunoassay format provides a means to directly measure the form of PSA that is known to increase in the serum of patients with CaP *(13, 14)*. Previous data have shown that the use of complexed PSA may improve the sensitivity for CaP detection *(13)*, and several studies have found that the ratio of PSA-ACT/total PSA showed an increase in the area under the curve by ROC analysis compared with total PSA, whereas the

use of complexed PSA alone showed no improvement by ROC analysis *(26, 28)*.

Numerous studies have shown that the discrimination of men with CaP from those with benign prostate disease could be improved through the implementation of a ratio of free and total PSA. This approach has several problems. The first is that free PSA is not stable in serum, probably as a result of complexation of free PSA with α-2-macroglobulin, which is nonreactive in current PSA assays. Free PSA is also present in very low concentrations in serum, particularly in the asymptomatic population, which may make accurate measurement of serum free PSA concentrations difficult. In addition, the use of a free PSA measurement requires that patient samples be "reflex tested" if the total PSA value falls within a predetermined "gray zone", but the gray zone is not well defined and has been variably suggested as 4–10 μg/L (4–10 ng/mL) *(22, 29)*, 3–10 μg/L (3–10 ng/mL) *(30)*, 4–25 μg/L (4–25 ng/mL) *(31)*, and >4 μg/L (>4 ng/mL) *(32)*. For these reasons, the clinical adaptation of the free-to-total PSA ratio is complicated and may delay implementation of this approach as a standard of care for the early detection of CaP.

Our results show that the amount of PSA in complex with protease inhibitors increases in patients with cancer compared with patients with benign prostate pathology (Fig. 4). Therefore, the measurement of cPSA could prove useful in improving the specificity of PSA testing for CaP. Any assay aimed toward improvement of specificity in CaP screening must maintain current levels of sensitivity. We therefore determined the Decision Limit for the cPSA assay that would provide equivalent sensitivity to total PSA at 4 μg/L (4 ng/mL), using this limited patient cohort. This limit was found to be 3.75 μg/L (3.75 ng/mL); it is to be expected that this value may be adjusted as larger studies are conducted. Alternatively, it may be possible to choose a Decision Limit such that both sensitivity and specificity are optimized. The development of an automated immunoassay that measures complexed PSA accurately will make it possible to answer such questions and to investigate the clinical utility of the complexed fraction of PSA in serum. Studies are currently underway to address these questions and to compare the utility of complexed PSA measurement with that of free PSA.

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