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In Vitro Characterization of Radiolabeled Monoclonal Antibodies Specific for the Extracellular Domain of Prostate-specific Membrane Antigen[1]

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ABSTRACT

Prostate-specific membrane antigen (PSMA) is a well-characterized cell surface antigen expressed by virtually all prostate cancers (PCas). PSMA has been successfully targeted *in vivo* with ^{111}In-labeled 7E11 monoclonal antibody (mAb; ProstaScint; Cytogen, Princeton, NJ), which binds to an intracellular epitope of PSMA. This work reports the *in vitro* characterization of three recently developed mAbs that bind the extracellular domain of PSMA ($PSMA_{ext}$). Murine mAbs J415, J533, J591, and 7E11 were radiolabeled with ^{131}I and evaluated in competitive and saturation binding studies with substrates derived from LNCaP cells. J415 and J591 were conjugated to 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*-tetraacetic acid labeled with ^{111}In. The uptake and cellular processing of these antibodies were evaluated in viable LNCaP cells. All four mAbs could be labeled with ^{131}I up to a specific activity of 350 MBq/mg with no or little apparent loss of immunoreactivity. Competition assays revealed that J415 and J591 compete for binding to $PSMA_{ext}$ antigen. J533 bound to a region close to the J591 binding epitope, but J533 did not interfere with J415 binding to PSMA. mAb 7E11 did not inhibit the binding of J415, J533, or J591 (or *vice versa*), consistent with earlier work that these latter mAbs bind $PSMA_{ext}$ whereas 7E11 binds the intracellular domain of PSMA. Saturation binding studies demonstrated that J415 and J591 bound with a similar affinity (K_ds 1.76 and 1.83 nM), whereas J533 had a lower affinity (K_d, 18 nM). In parallel studies, all four mAbs bound to a similar number of PSMA sites expressed by permeabilized cells (1,000,000–1,300,000 sites/cell). In parallel studies performed with viable LNCaP cells, J415, J533, and J591 bound to a similar number of PSMA sites (*i.e.*, 600,000–800,000 sites/cell), whereas 7E11 bound only to a subpopulation of the available PSMA sites (95,000 sites/cell). This apparent binding of 7E11 to viable cells can be accounted for by a 5–7% subpopulation of permeabilized cells produced when the cells were trypsinized and suspended. Up to five DOTA chelates could be bound to either J415 or J591 without compromising immunoreactivity. A comparison of the cellular uptake and metabolic processing of the ^{131}I- and ^{111}In-labeled antibodies showed a rapid elimination of ^{131}I from the cell and a high retention of ^{111}In. All four mAbs recognized and bound to similar numbers of PSMAs expressed by ruptured LNCaP cells (*i.e.*, the exposed intracellular and extracellular domains of PSMA). By comparison to J415 and J591, J533 had a lower binding affinity. Both J415 and J591 recognized and bound to the same high number of PSMAs expressed by intact LNCaP. By contrast, 7E11 bound to fewer sites expressed by intact LNCaP cells (*i.e.*, the exposed extracellular domain of PSMA). Both J415 and J591 are promising mAbs for the targeting of viable PSMA-expressing tissue with diagnostic and therapeutic metallic radionuclides.

INTRODUCTION

PCa[3] is the most frequently diagnosed cancer and the second most common cause of cancer mortality in United States males (1). Many groups have studied mAbs for *in vivo* diagnosis and therapy of PCa (3–8), but the only successful application to date has been the targeting of PSMA for *in vivo* imaging. PSMA is a type II membrane protein that is expressed by virtually all PCas (9, 10). Unlike other prostate-related antigens, such as prostate-specific antigen, prostatic acid phosphatase, and prostate secretory protein, PSMA is an integral membrane protein, and therefore, it is not appreciably released into the circulation. PSMA expression has been shown to be up-regulated in both poorly differentiated (11), advanced PCas (9) and after androgen-deprivation therapy (12). Interestingly, PSMA is expressed on the tumor vascular endothelium of other carcinomas and sarcomas (13, 14) but not on normal vascular endothelium, making it also potentially useful as an antibody-mediated diagnostic and therapeutic target across the full spectrum of solid tumors.

Currently, an ^{111}In-labeled form of the 7E11 murine mAb is approved by the Food and Drug Administration (Prostascint) for the clinical detection of recurrent and metastatic prostate cancer in soft tissue (15). 7E11 mAb binds to the intracellular portion (NH_2 terminus) of the PSMA antigen and, as such, does not bind viable cells (13, 14). It is believed that successful imaging with Prostascint results from mAb binding to antigen exposed in dead or dying cells within some tumor sites (16, 17). Early clinical trials using ^{90}Y-labeled 7E11 resulted in no objective or biochemical (prostate-specific antigen) remissions (7).

Recently, a series of mAbs to $PSMA_{ext}$ has been characterized and reported (13, 14, 18). In this current study, we report on the *in vitro* evaluation of radiolabeled forms of these antibodies against $PSMA_{ext}$ and the selection of interesting candidates for *in vivo* evaluation of their diagnostic and therapeutic potential.

MATERIALS AND METHODS

Murine mAbs J415, J533, and J591 were produced as described earlier (13). Purified 7E11 was generously provided by Dr. Gerald P. Murphy (Pacific Northwest Research Foundation, Seattle, WA). ^{131}I and ^{111}In were purchased from Norton International (Kanata, Ontario, Canada). ^{90}Y was purchased from New England Nuclear (Boston, MA). DOTA was purchased from Macrocyclics, Inc. (Richardson, TX).

LNCaP cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640, supplemented with 10% FCS, at a temperature of 37°C in an environment containing 5% CO_2. Prior to use, the cells were trypsinized, counted, and suspended in serum-free medium. LNCaP cells were permeabilized by adding methanol at −80°C to the cells. The cells were maintained at −20°C for 20 min before the methanol was removed, and the cells were rehydrated by washing four times with PBS (with 5 mM Ca^{2+} and 5 mM Mg^{2+}) over 20 min.

Cell membranes were prepared by lysing the cells with a Polytron in a

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[3] The abbreviations used are: PCa, prostate cancer; mAb, monoclonal antibody; PSMA, prostate-specific membrane antigen; DOTA, 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*-tetraacetic acid; BSA, bovine serum albumin; TLC, instant TLC; HPLC, high-performance liquid chromatography; DTPA, diethylenetriaminepentaacetic acid.

hypotonic buffer [1 mM Na_2CO_3 (pH 7.4) with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). Large fragments were removed by centrifuging at 2000 × *g*. The supernatant was centrifuged at 150,000 × *g* for 2 h, and the pelleted membranes were resuspended in PBS, aliquoted, and frozen at −70°C until required.

Radioiodination

The four murine mAbs were radiolabeled with ^{131}I using the Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) method (19). Briefly, 10-ml glass tubes were coated with 50 μg of Iodogen by adding 100 μl of a 0.5 mg/ml solution of Iodogen (Pierce, Rockford, IL) in chloroform. The chloroform was removed by blowing a gentle stream of sterile nitrogen into the tube for 30 min before the tubes were sealed and stored in the dark. The iodination reaction was initiated by adding between 4 and 40 MBq of ^{131}I (0.01 M NaOH) to 0.08 mg of mAb in 0.1 ml of ice-cold PBS. This reaction mixture was allowed to react for 5 min on ice before being loaded onto a 10-ml Biogel-P6 column (Bio-Rad Laboratories, Hercules, CA) equilibrated with 1% BSA in PBS. Once the reaction mixture was loaded onto the column, it was washed with 2 ml of 1% BSA PBS before the main ^{131}I-labeled mAb fraction was eluted with 2 ml of 1% BSA PBS. The amount of free iodine in the ^{131}I-labeled mAb preparations was evaluated using instant TLC with a silica gel impregnated glass fiber support and a mobile phase of isotonic saline. Briefly, a portion of the ^{131}I-labeled mAb was spotted on a 10-cm ITLC-SG strip (Gelman Sciences, Ann Arbor, MI) and developed in isotonic saline. Once the solvent front had reached the end of the strip, it was removed from the solvent and cut at an R_f of 0.5. The two portions were assayed for radioactivity, and the radiochemical purity determined using the following equation: radiochemical purity = activity between R_f 0 and 0.5/total activity in strip.

Antibody Conjugation

J415 and J591 antibodies were modified with DOTA by an analogous method to that used by Lewis *et al.* (20). This method uses the direct coupling of one of the four carboxylic acid groups of DOTA to the primary amines present in the protein structure (Fig. 1). Twenty-five mg of antibody were concentrated in a M_r 30,000 Microsep centrifugal concentrator (Pall Filtron, Northborough, MA) and washed with 5 × 4 ml of 1% DTPA (pH 5.0) over a period of 24 h. The antibody buffer was then changed to 0.1 M phosphate (pH 7.0) using the same centrifugal technique. An active ester of DOTA was created by dissolving 146 mg of DOTA (0.361 mmol) and 36 mg of *N*-hydroxysuccinimide (0.313 mmol) in 2 ml of water and adjusting the pH to 7.3 with NaOH, prior to the addition of 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. This reaction mixture was cooled on ice for 1 h before being added to the J591 solution. The resultant DOTA-antibody conjugate was separated from the excess DOTA and other reactants by repeated washing with 0.3 M NH_4OAc (20 × 4 ml) and centrifugal concentration.

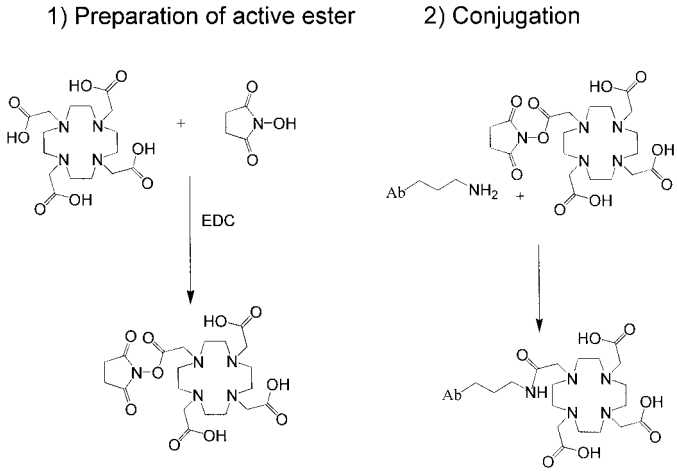


Fig. 1. Two-step conjugation of DOTA to free amines displayed by either J415 or J591. The first step used *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimine to create an active ester with DOTA. In the second step, the unpurified active ester is allowed to react with the monoclonal antibody.

Assay of Binding Site Number

DOTA-J591 conjugate concentration was assayed by determining the UV absorption at 280 nm. Two 50-μl aliquots of DOTA-J591 were mixed with either 20 or 40 μl of a 1.30 mM solution of $InCl_3$ (0.01 M HCl) spiked with a tracer amount of ^{111}In. The mixture was incubated at 37°C for 16 h and then analyzed by ITLC, using a silica gel-impregnated glass fiber 10-cm strip (ITLC-SG; Gelman) and an eluant of 1% DTPA (pH 6.0). Antibody-bound activity remains at the origin, and free In^{3+} moves with the solvent front as an $[In\text{-}DTPA]^{2-}$ complex. The relative amounts of In^{3+} and In-DOTA-J591 were determined by cutting the ITLC strip at a R_f of 0.5 and counting the two halves with a Na(Tl)I detector. The number of binding sites was calculated by considering the molar reaction ratio between In and DOTA-mu-J591, and the observed ratio of ^{111}In and ^{111}In-DOTA-mu-J591 was detected.

^{111}In and ^{90}Y Labeling of DOTA Conjugate

Radiolabeling of DOTA-J591 with ^{111}In was achieved by adding the radionuclide (in dilute HCl) to the ammonium acetate-buffered DOTA-J591. Briefly, a mixture composed of 20 μl of $^{111}InCl_3$ (300 MBq), 0.01 M HCl, and 400 μl of DOTA-J591 (4 mg/ml; 0.3 M NH_4OAc, pH 7) was allowed to react at 37°C for 20 min. The reaction mixture was then separated on a 20-ml Biogel-P6 column equilibrated with 4 × 10 ml of sterile 1% HSA in PBS. After the reaction mixture was loaded onto the column, it was washed with an additional 5 ml of 1% HSA PBS before the main ^{111}In-DOTA-J591 fraction was eluted with 3 ml of 1% HSA PBS. A similar procedure was used for radiolabeling with ^{90}Y, but an incubation time of 5 min was used, and the labeling mixture included 50 mM ascorbic acid.

Free ^{111}In in the radiolabeled DOTA-J591 preparations was determined using the ITLC method with a silica gel-impregnated glass fiber support and a mobile phase of 1% DTPA (pH 5.5). A portion of the radiolabeled DOTA-J591 was spotted on a 10-cm ITLC-SG strip and developed in 1% DTPA (pH 5.5). Once the solvent front had reached the end of the strip, it was removed from the solvent and cut at a R_f of 0.5. The two portions were assayed for radioactivity, and the radiochemical purity was determined using the equation described earlier.

Chelate Stability Studies

^{111}In-labeled DOTA-J591 and DTPA-7E11 were mixed with an equal volume of 50 mM DTPA and maintained at 37°C. Periodically, samples were removed and spotted on a 10-cm ITLC-SG strip and developed in 0.9% NaCl. Once the solvent front had reached the end of the strip, it was removed from the solvent and cut at a R_f of 0.5. The two portions were assayed for radioactivity, and the amount of intact chelate was determined using the equation described earlier.

Binding Studies

Immunoreactivity. The immunoreactivity of the ^{131}I- and ^{111}In-labeled mAb preparations was assessed by the method of Lindmo *et al.* (21), which extrapolates the binding of the radiolabeled antibody at an infinite excess antigen. Briefly, six test solutions were prepared (in duplicate) and contained 20,000 cpm of the radioiodinated antibody, and increasing amounts of membranes were prepared from LNCaP cells in a total test volume of 250 μl of PBS (0.2% BSA, pH 7.4). The solutions were incubated at 37°C for 45 min prior to being filtered through a glass membrane filter and washed with ice-cold 10 mM Tris-0.9% NaCl buffer. Filters were counted in a gamma counter with standards representing the total radioactivity added. Data were then plotted as the reciprocal of the substrate concentration (X axis) against the reciprocal of the fraction bound (Y axis). The data were then fitted according to a least squares linear regression method using Origin software (Microcal Software, Inc., Northampton, MA). The Y intercept gave the reciprocal of the immunoreactive fraction. A similar method using intact or permeated LNCaP cells and centrifugational isolation of the cells gave the same results.

Competitive Binding Studies. Competitive binding studies were performed with each of the radioiodinated antibodies and the four unlabeled antibodies using either LNCaP tumor sections or membranes derived from LNCaP tumors. Acetone fixed and frozen 10-μm tumor sections were soaked in Tris buffer [170 mM (pH 7.4), with 2 mM $CaCl_2$ and 5 mM KCl] for 15 min and then washed with Tris buffer (170 mM, pH 7.4). The sections were then

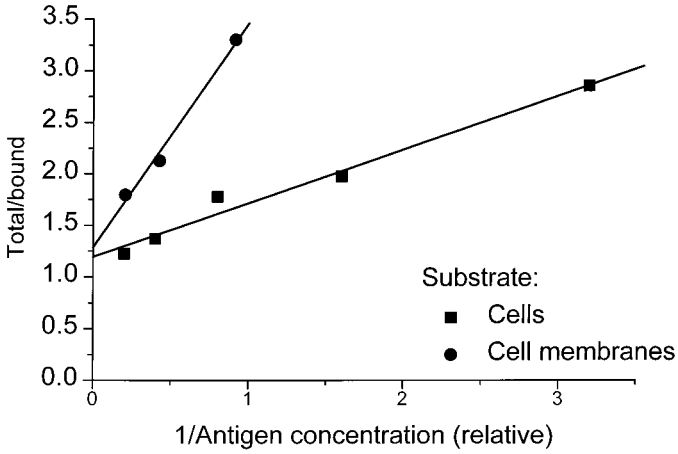


Fig. 2. Lindmo immunoreactivity testing of radiolabeled antibodies. Twenty thousand cpm of radiolabeled antibody is incubated with increasing amounts of LNCaP cell membranes at 37°C for 45 min. The membranes are then isolated by filtration through a glass fiber filter and then counted in a gamma counter. Total/bound data are then plotted as a function of the reciprocal antigen concentration. The *Y* intercept gives the reciprocal of the immunoreactivity. The assay, when performed with either intact LNCaP cells (■) or LNCaP cell membranes (●), gives the same immunoreactivity of ~80% for this labeled mAb.

incubated with the radioiodinated antibodies in the presence of 100 nM concentrations of each of the unmodified mAbs for 1 h at 4°C. Sections were washed three times with PBS (0.2% BSA) and once with Tris buffer (170 mM, pH 7.4) prior to being fixed with acetone and exposed with BioMax film (Kodak). The assay using the membranes typically used 50 μg of membranes, 10 fmol of iodinated antibody, and amounts of competing antibody from 0.25 fmol to 25 pmol in a 250-μl volume of PBS (0.2% BSA). Membranes were isolated as described above, and data were analyzed by a least squares regression method and Origin software (Microcal Software, Inc.) was used to determine the IC_{50}s.

Saturation Binding Studies. Saturation binding studies were performed with each of the radiolabeled antibodies using substrates of intact and permeated LNCaP cells. Briefly, 10 test solutions were prepared (in duplicate) and they contained increasing amounts of the radioiodinated antibodies, 500,000 LNCaP cells in a total volume of 250 μl of PBS (0.2% BSA, pH 7.4). The solutions were incubated at 4°C for 1 h and centrifuged and washed twice with ice-cold PBS (0.2% BSA). For each concentration of radiolabeled antibody, nonspecific binding was determined in the presence of 100 nM of the unmodified antibody. The data were analyzed with a least squares regression method (Origin; Microcal Software, Inc.) to determine the K_d and B_{max} values, and a Scatchard transformation was performed.

Internalization and Cellular Processing of J415 and J591

LNCaP cells were plated in 8-cm^2 Petri dishes and allowed to grow until confluent. One μCi of either the ^{131}I- or ^{111}In-labeled forms of J415, J591, or 7E11 (~0.1–0.2 μg) were added to cells and allowed to incubate for 1 h. The medium was then removed, and the cells were washed once with fresh media. One ml of fresh medium was added, and the cells were incubated for up to 2 days at 37°C. Triplicate samples were periodically removed, and the medium was isolated. Surface bound activity was stripped and collected with an ice-cold acid wash (100 mM acetic acid, 100 mM glycine, pH 3.0). The cells were then treated with 1 ml of a 1% solution of Triton X-100 (containing 5 μg/ml each of antipain, pepstatin, and leupeptin as well as 1 mM phenylmethylsulfonyl fluoride) and kept at on ice for 20 min. The resultant suspension was then centrifuged, and the three samples were counted with a gamma counter. The medium and supernatants were also analyzed by ITLC and size exclusion HPLC to determine the amounts of free iodide produced or the size of the radioactive species created.

RESULTS

Radiolabeling and Quality Control. The radioiodination yield for the four mAbs was typically 70–80%, and the amounts of free iodine in the purified mAbs was <0.3%. Specific activities of 350 MBq/mg were routinely achieved. The immunoreactivities of the ^{131}I-labeled mAbs were determined by extrapolating the binding of a fixed amount of ^{131}I-labeled mAb to an infinite amount of PSMA (Lindmo method; Fig. 2). This method gave immunoreactivities of >75% for all mAbs tested. When labeling conditions were increased to produce specific activities >350 MBq/mg, the immunoreactivity was compromised.

An average of five DOTA molecules could be randomly conjugated to J591 and J415, with little apparent loss of immunoreactivity. Conjugation of an average of eight DOTA molecules to J591 resulted in a 20% reduction in immunoreactivity. A 90% incorporation of ^{111}In could be achieved within 15 min. A 90% incorporation of ^{90}Y could be achieved within 5 min. Using the DOTA-J591 conjugate with an average of five DOTA molecules attached, specific activities of 280 MBq ^{111}In/mg DOTA-J591 and 360 MBq ^{90}Y/mg DOTA-J591 were achieved.

^{111}In-Chelate Stability Studies. A direct comparison of the chelate stability of ^{111}In-DTPA-7E11 and ^{111}In-DOTA-J591 showed that ^{111}In was lost from DTPA-7E11 with an apparent half-life of 11 h, whereas the DOTA chelate had an apparent half-life exceeding 1000 h (Fig. 3).

Competitive Binding Studies: Membranes. Radiolabeled J415 could be displaced from binding to LNCaP cell membranes by both J415 and J591 but not J533 (Fig. 4*A*). The J415 mAb had an mean IC_{50} of 1.5 nM (±0.9; $n = 6$), and J591 had a mean IC_{50} of 6.6 nM (±4.5; $n = 6$). Similarly, ^{131}I-labeled J533 could be displaced by J533 and J591 but not by J415 (Fig. 4*B*). In these studies, J533 had a mean IC_{50} of 2.3 nM (±1.5; $n = 3$), and J591 had a mean IC_{50} of 1.7 nM (±1.3; $n = 3$). Finally, ^{131}I-labeled J591 could be displaced by J415, J533, and J591 (Fig. 4*C*). The observed IC_{50}s were 1.3 nM (±0.9; $n = 6$) for J415, 7.7 nM (±5.5; $n = 6$) for J533, and 3.1 nM (±1.5; $n = 6$) for J591. The 7E11 mAb did not inhibit the binding of J415, J533, or J591 (or *vice versa*). These data are consistent with earlier data (11) that J415, J533, or J591 bind to the extracellular domain of PSMA, whereas 7E11 binds to the intracellular domain of PSMA (13).

Saturation Binding Studies. The saturation binding curves generated were characteristic of high affinity binding of an antibody to a single class of antigen. These studies, performed with intact LNCaP cells (Fig. 5), demonstrated that J415 and J591 bound with a similar

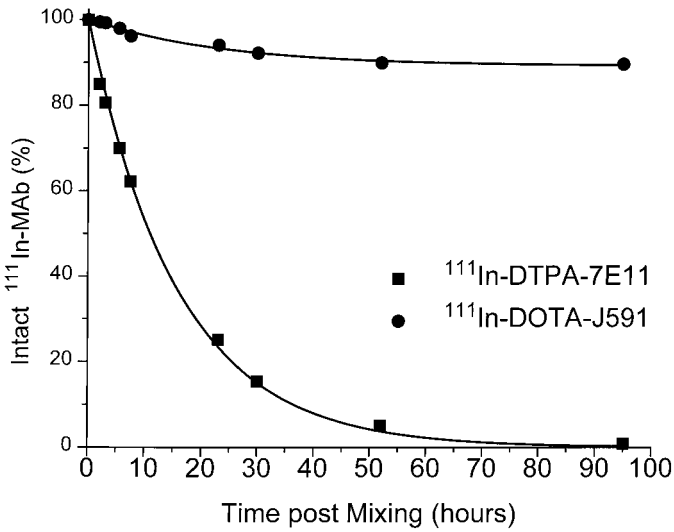
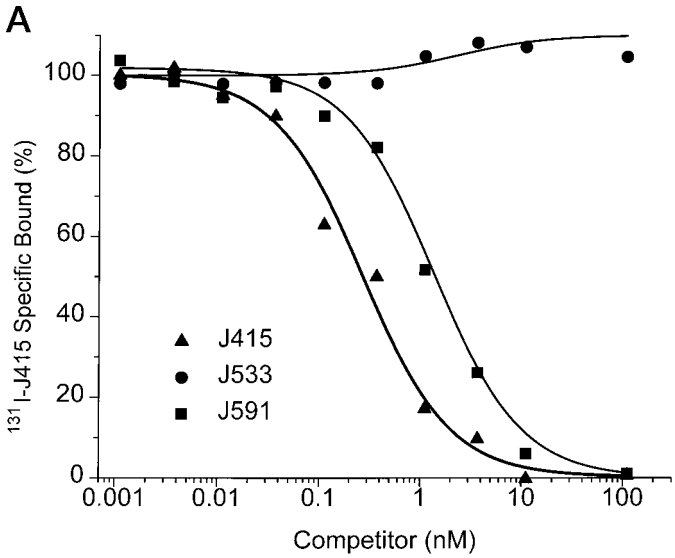
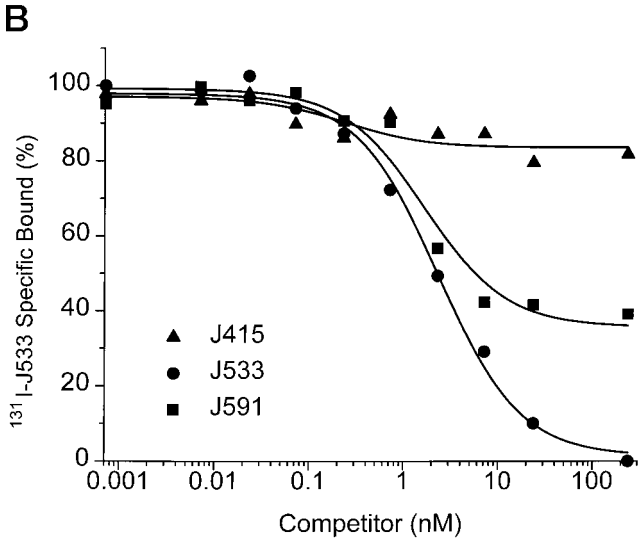


Fig. 3. Stability of ^{111}In-labeled mAbs. The ^{111}In-labeled mAbs were mixed with 50 mM DTPA, and samples were removed and analyzed by ITLC over the next 4 days. ^{111}In-DOTA-J591 (●) had an apparent half-life of >1000 h, and ^{111}In-DTPA-J591 (■) had an apparent half-life of 11 h.





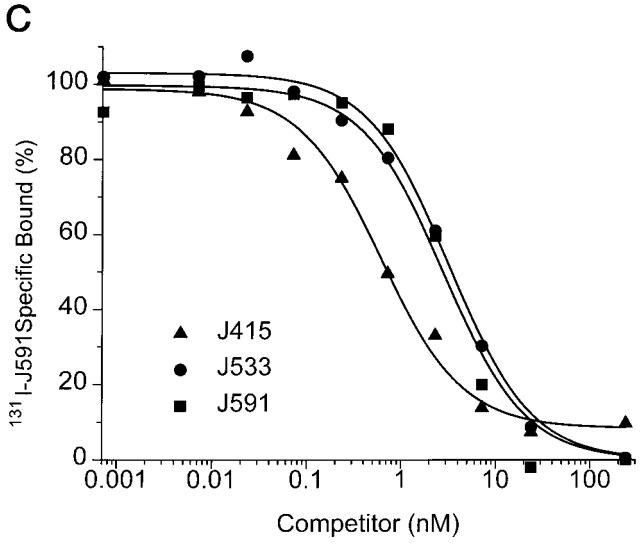


Fig. 4. Displacement binding of ^{131}I-labeled J415 (*A*), ^{131}I-labeled J533 (*B*), and ^{131}I-labeled J591 (*C*) to LNCaP cell membranes. The radioiodinated mAbs are incubated a fixed amount of LNCaP cell membranes in the presence of increasing concentrations of either J415 (▲), J533 (●), or J591 (■) at 37°C for 45 min. The membranes are then isolated by filtration through a glass fiber filter and then counted in a gamma counter. The amount of specific iodinated mAb bound is then plotted as a function of the increasing concentrations of the competing antibodies.

affinity (K_ds 1.76 ± 0.69 and 1.83 ± 1.21 nM), whereas J533 had a lower affinity (K_d 18 ± 5 nM). In parallel studies, all four mAbs bound to a similar number of PSMA sites expressed by permeabilized cells (1,000,000–1,300,000 sites/cell). In parallel studies performed with viable LNCaP cells, J415, J533, and J591 bound to a similar number of PSMA sites (*i.e.*, 600,000–800,000 sites/cell). In contrast, 7E11 specifically bound to only 10–15% of the PSMA sites expressed by apparently intact LNCaP Cells (K_d, 6.69 nM); but when the cells were deliberately ruptured (Fig. 6), 7E11 bound to a similar number of antigen sites as the other three mAbs. In parallel studies, using ^{131}I-labeled J591, permeabilized cells expressed about twice the amount of PSMA as intact LNCaP cells, suggesting that not all available PSMA is simultaneously expressed on the cell surface.

Internalization and Cellular Processing of J415 and J591. Both ^{131}I-labeled J415 and J591 demonstrated a poor cellular retention of

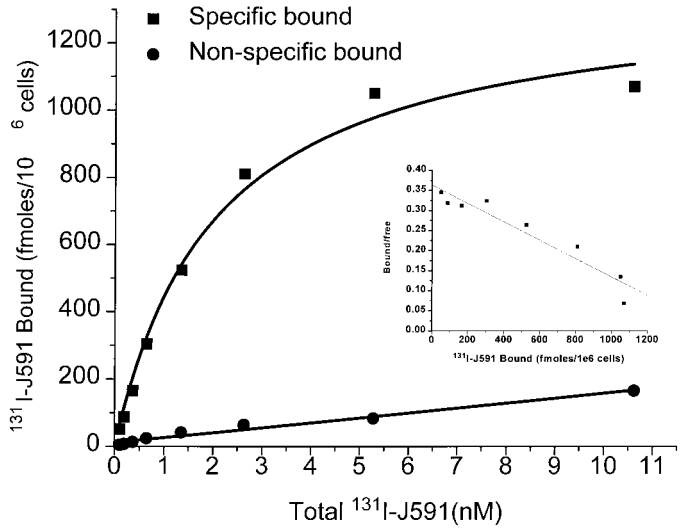


Fig. 5. Saturation binding of ^{131}I-labeled J591 to LNCaP cells. Increasing concentrations of ^{131}I-labeled J591 were incubated with intact LNCaP cells on ice for 60 min. Nonspecific binding (●) was determined in the presence of 100 nM unlabeled J591. Bound activity was isolated by centrifuging the cells and washing them twice with ice-cold buffer. *Inset*, Scatchard plot of the same data.

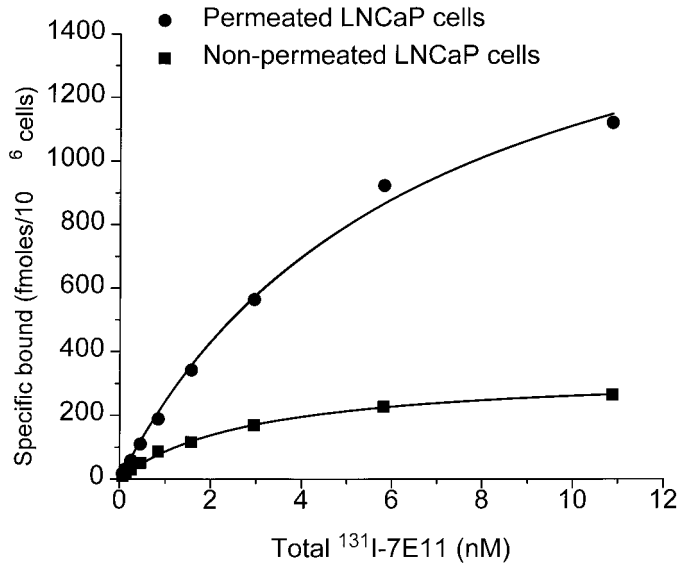


Fig. 6. Saturation binding of ^{131}I-labeled 7E11 to intact and ruptured LNCaP cells. Increasing concentrations of ^{131}I-labeled 7E11 were incubated with either intact (●) or permeabilized (■) LNCaP cells on ice for 60 min. Nonspecific binding was determined in the presence of 100 nM unlabeled 7E11. Bound activity was isolated by centrifuging the cells and washing them twice with ice-cold buffer.

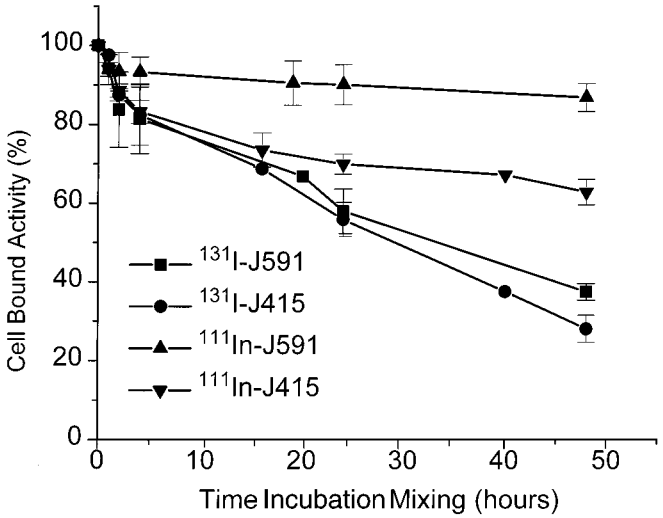


Fig. 7. LNCaP cell retention of radioiodinated J415 and J591. Petri dishes (8 cm^2) with confluent LNCaP cells were incubated with 37 KBq of either ^{131}I-labeled J591 (■), ^{131}I-labeled J415 (●), ^{111}In-labeled J591 (▲), or ^{111}In-labeled J415 (▼). After 1 h at 37°C, the medium was removed, and the cells were washed once with fresh medium. One ml of fresh medium was added, and the cells were incubated for up to 2 days at 37°C. At various time points, the location and form of the radioactivity was determined. *Bars,* SD.

radioactivity (Fig. 7). For both mAbs, a biexponential curve fit of the data showed that ~10% of the radioactivity was released from the cells with an apparent half-life of 1 h, and the remaining 90% was released into the medium with apparent half-lives of 31 and 38 h for J415 and J591, respectively. In parallel studies, ^{131}I-labeled J415 consistently showed a faster release of radioactivity than ^{131}I-labeled J591. Little or no activity (<1%) was associated with the Triton X-100 (or NaOH) insoluble cell pellet. Analysis of the Triton X-100 soluble fractions indicated that there were no appreciable amounts of free ^{131}I present (<1%). HPLC and TLC analysis of the culture medium showed that a large iodinated species, which corresponded to the same size as the intact mAbs, was being released from the cells, but this never amounted to >10% of the total activity, and after 4–6 h, no further release of this radioactive species was observed. The predominant metabolite of the iodinated mAbs found in the cell medium had the same HPLC and TLC elution profile as free $^{131}I^-$. Several studies compared ^{131}I-labeled J591 and DOTA-J591, and no significant differences in the retention of ^{131}I by the cells were noted between the two forms of the same mAb. In all of the studies performed, no increase in cell death was noted as compared with the control groups that received no radiolabeled antibodies.

In comparison to the iodinated mAbs, the ^{111}In-labeled DOTA-J415 and DOTA-J591 demonstrated a high cellular retention of radioactivity (Fig. 7). For ^{111}In-DOTA-J415, a biexponential curve fit of the data showed that ~20% of the radioactivity was released from the cells with an apparent half-life of 2 h, and the remaining 80% was released into the medium with an apparent half-life of 160 h. For ^{111}In-DOTA-J591, the cellular release of ^{111}In species was much slower, and a biexponential curve fit of the data showed that about 5–10% of the radioactivity was released from the cells with an apparent half-life of 1 h, and the remaining 90–95% was being released into the medium with an apparent half-life of 520 h. Little or no activity (<1%) was associated with the Triton X-100 (or NaOH) insoluble cell pellet. HPLC and TLC analysis of the cell medium showed that a large ^{111}In species, which corresponded to the same size at the intact mAbs, was being released from the cells, but this never amounted to >10% of the total activity, and after 4–6 h, no further release of this radioactive species was observed. For both J415 and J591, two main ^{111}In-labeled metabolites were observed in the medium. Analysis of the cell-associated radioactivity (Fig. 8) demonstrated the rapid formation of two groups of metabolites (based on molecular size). One group of metabolites achieved a maximum concentration after 1–2 h, after which it began to steadily decline. The second group of metabolites, however, demonstrated an ever-increasing intracellular concentration. This second metabolite did not behave the same as $^{111}In^{3+}$ (HPLC or TLC), but rather it had a similar molecular weight as an ^{111}In-DOTA or an ^{111}In-DOTA-amino acid fragment. The first metabolite had a molecular weight between that of the intact mAb and the second metabolite (M_r ~10,000–30,000).

The uptake rates of ^{111}In-labeled J415, J591, and 7E11 by LNCaP cells showed a similar initial uptake rate for J415 and J591, which was 10–20 times faster than that of 7E11 (Fig. 9). However, by 4 h after the addition of the radioactivity, the cells treated with ^{111}In-DOTA-J591 have incorporated and retained more activity than those treated with ^{111}In-DOTA-J415.

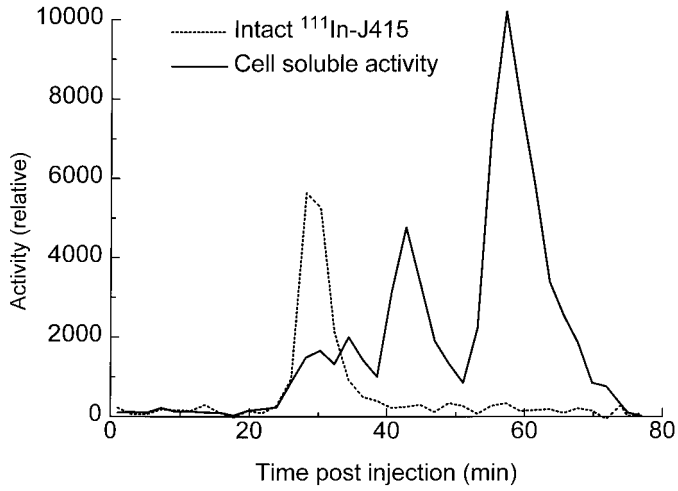


Fig. 8. HPLC chromatograms of ^{111}In-DOTA-J415 and radioactivity recovered from LNCaP cells at 48 h after incubation. The intact ^{111}In-DOTA-J415 elutes at 28 min after injection, and two main metabolites elute at 43 and 57 min after injection, respectively.

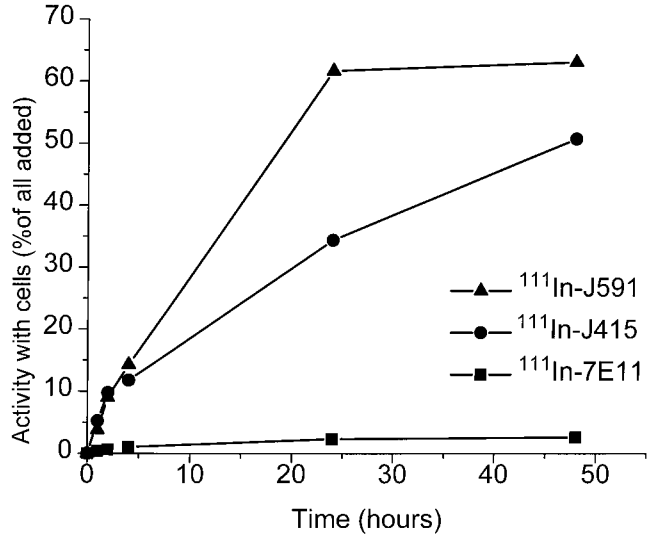


Fig. 9. Incorporation of ^{111}In-labeled cells by LNCaP cells. Petri dishes (8 cm^2) with confluent LNCaP cells were incubated with 37 KBq of either the ^{111}In-DOTA-J415 (●), ^{111}In-DOTA-J591 (▲), or ^{111}In-DTPA-7E11 (■). The samples were incubated at 37°C, and at various time points the location and the amount of cell-associated radioactivity were determined.

Table 1 *Inhibition constants for unlabeled J415, J533, J591, and 7E11 for the binding of ^{131}I-labeled J415, ^{131}I-labeled J533, and ^{131}I-labeled J591 binding to PMSA expressed by LNCaP cell membranes*

	IC_{50}s for displacing mAb (nM)			
Iodinated mAb	J415	J533	J591	7E11
^{131}I-labeled J415	1.5 ± 0.9	ND[a]	6.6 ± 4.5	ND
^{131}I-labeled J533	ND	2.3 ± 1.5	1.7 ± 1.3	ND
^{131}I-labeled J591	1.3 ± 0.9	7.7 ± 5.5	3.1 ± 1.5	ND

[a] ND, not displaced.

DISCUSSION

To understand the characteristics of and to select the best imaging/therapeutic agent, we studied binding characteristics and retention rates of the variously labeled mAbs using the LNCaP cell line, which expresses PMSA (Table 1).

The initial labeling of the three mAbs with ^{131}I, up to a specific activity of 350 MBq/mg, resulted in little or no apparent loss of immunoreactivity. Similarly, the conjugation of up to an average of five DOTA chelates per mAb enabled specific activities of up to 280 MBq ^{111}In/mg DOTA-J591 with no apparent loss of immunoreactivity. Site-specific modification of the antibody is sometimes required when this type of random DOTA coupling results in loss of immunoreactivity attributable to the presence of a lysine residue in the antigen binding domain. High specific activities are often required for accurate mAb characterization and particularly when large amounts of the radiotherapeutic agent are administered to a patient. J451 and J591 could be modified with sufficient DOTA to produce high specific activity ^{111}In- and ^{90}Y-labeled mAbs for both *in vitro* binding studies and eventual *in vivo* studies.

Early approaches to labeling mAbs with radiometals used DTPA, which in its dicyclic anhydride form could be conveniently coupled to mAbs (22). Unfortunately, this simple coupling chemistry produced a more labile chelate than bifunctional forms of the same unconjugated DTPA chelator (23). Macrocylic chelators have shown even higher kinetic stability (24), but they are even more time consuming to chemically synthesize (25). DOTA can be coupled directly to mAbs using simple chemistry and commercially available materials (20).

The reaction kinetics for ^{111}In and DOTA are longer than for DTPA, but an incubation period of 15 min can give high labeling yields. The DOTA chelator was immensely superior to DTPA in its ability to tightly chelate ^{111}In in the presence of an excess of competing ligand. This is in agreement with other studies (20, 26) and underlies the importance of using stable chelates with mAbs that can stay in circulation for prolonged periods of time in the presence of competing ligands (*e.g.*, transferrin). The higher stability of the ^{111}In-labeled DOTA complex relative to the ^{111}In-labeled DTPA complex also applies for the ^{90}Y complex (20, 26) and is an important prerequisite for radiolabeled mAbs used for either diagnosis or therapy because optimal tumor:nontumor ratios are often achieved after 2–4 days. Because ^{111}In-DOTA-J591 is stable to DTPA competition, it enables nonspecifically bound ^{111}In to be removed by challenging with DTPA and a simple column separation to yield a highly pure radiopharmaceutical.

High binding affinity between the mAb and the target antigen is another prerequisite to *in vivo* targeting of tumor antigens. The binding studies with the iodinated mAbs showed that two of three of these mAbs against $PSMA_{ext}$ (*i.e.*, J415 and J591) and 7E11 have similar nanomolar binding affinities. The use of intact and ruptured cells showed clearly that ^{131}I-labeled 7E11 binds to the intracellular domain of PMSA, consistent with other reports (13, 17). There was some binding of 7E11 to "intact" LNCaP cells, but that could be explained by the presence of a small population of cells ruptured during the trypsinization of the cells from the cell culture flasks and subsequent handling during resuspension. Because J591 recognized and specifically bound to twice the number of PSMA sites in permeabilized cells as opposed to intact cells, this suggests that only 50% of all cellular PSMA is exposed extracellularly. Also, 10–15% binding of 7E11 could be explained by the presence of a population of 5–7% of permeabilized cell in the "intact" cell preparation. This was confirmed in other studies that examined the binding and cellular uptake of the mAbs with plated LNCaP cells and showed a 7E11 uptake of 3–4% that of J415 and J591. This quantitative difference seen might explain why one group claims that the 7E11 binds to apparently intact LNCaP cells (27), whereas other groups report no such binding (13, 17, 18). Additionally, the Barren study (27) used cells that were scrapped from the monolayer, a procedure known to create significant cell rupture.

Effective systemic targeting of tumors has been achieved with both iodinated and metallochelated antibodies. A significant factor in selecting between the two approaches is in whether the antibody is internalized. In the case of an internalizing antibody, directly iodinated antibodies are metabolized within the cell, and the main metabolites of iodide and iodotyrosine are then freely released from the cell. Conversely, the metal chelate-labeled antibody is metabolized to leave a chelate-amino acid fragment that is typically not released from the cell and is trapped for further degradation. This effect can produce vastly different residence times for two differently labeled forms of the same antibody (28). In the LNCaP cell model, PSMA is known to be an internalizing surface bound glycoprotein (18). These studies clearly support the notion that metallolabeled mAbs are superior for cells expressing internalizable PSMA and that metabolites from the DOTA-mAb conjugate are not appreciably released from LNCaP cells.

In conclusion, both J415 and J591 have similar nanomolar affinities to PSMA as 7E11. Similarly, these two mAbs are far more readily bound and were internalized by live LNCaP cells than 7E11. The ^{111}In-labeled DOTA conjugates are able to associate more radioactivity with LNCaP cells than the comparable iodinated forms. The ^{111}In-labeled DOTA conjugates are also more stable to loss of ^{111}In than DTPA-7E11. These findings make DOTA-J415 and DOTA-J591 attractive candidates for further evaluation as either diagnostic or radiotherapeutic agents in patients with various cancers that express PMSA.

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