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Mass Spectrometric Immunoassay for Parathyroid Hormone-Related Protein

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This paper describes a novel two-site peptide immunoassay using the isotope ^{14}C as the label and accelerator mass spectrometry as the detection system. A mouse monoclonal antibody (1A5) against the amino terminal region of human parathyroid hormone-related protein (PTHrP) was labeled with ^{14}C by growing the hybridoma cells in a miniPERM bioreactor in the presence of [U-^{14}C]L-leucine and [U-^{14}C]D-glucose. The antibody was purified from the culture media using protein G affinity chromatography. The purified ^{14}C-labeled antibody (^{14}C-1A5) fractions showed excellent correlation between the levels of radioactivity and binding activity for PTHrP. Using ^{14}C-1A5 as the detection antibody in a two-site immunoassay format for PTHrP1-141, a 16-kDa polypeptide, an analytic sensitivity of 10 pmol/L was achieved with a linear measurement range up to 1.3 nmol/L. Only ∼17 pCi/well (or 1.6 nCi/96-well microtiter plate) ^{14}C-1A5 was used, which is far below the limit (50 nCi/g) for disposal as nonradioactive waste. This study may serve as a model for the development of sensitive and "nonradioactive" immunoassays for peptides, including polypeptide tumor markers.

Despite the wide range of methodologies available in clinical laboratory testing, immunoassays are among the most widely used because of their sensitivity and specificity. In addition to applications in the diagnosis and management of various endocrine disorders and in clinical toxicology (drug screening and therapeutic drug monitoring), immunoassays also serve as important tools for the early diagnosis of cancer as well as for the monitoring of residual or recurrent cancer by the detection of tumor markers. There are two major immunoassay formats, competitive immunoassay and two-site immunoassay (or sandwich immunoassay). By using a pair of specific antibodies (an immobilized capturing antibody and a conjugated detection antibody) targeting different antigenic sites of the antigen, the two-site immunoassays can have higher analytical sensitivity than the competitive immunoassays. Monoclonal antibody technologies, including hybridoma[1] and phage display technology,[2] have made it possible to manufacture large amounts of specific antibodies at moderate cost, thereby allowing the two-site assay to be exploited and developed for a variety of bioactive targets.

Except for precipitation immunoassays, in which the resulting antigen−antibody complex in gel or liquid phase can be observed qualitatively by the naked eye or quantitatively with a detector, all other immunoassays involve the detection of labels. Since Yalow and Berson reported on the development of radioimmunoassay (RIA),[3] the radioisotope labeling system has allowed for the quantitative detection of trace levels of analytes.[4,5] RIA and immunoradiometric assays (IRMA) are advantageous for a number of reasons: precision and high sensitivity, ease of isotope conjugation, signal detection with little optimization, and stability against interference from the assay components such as plasma constituents. Conventional radioisotope detection methods, such as liquid scintillation counting (LSC) and autoradiography, use the radiation generated in the isotope decay process. The sensitivity of the detection assay correlates to the rate of decay or inversely to the half-life of the radioisotope. Commonly used short-life isotopes, such as ^{32}P (half-life, 14.3 days) and ^{125}I (half-life, 60 days), can be detected at attomole levels by LSC, thus rendering RIA/IRMA highly sensitive.[4,5] However, these short-lived, high-energy isotopes can pose substantial safety concerns in the clinical laboratory environment, and many strict regulatory requirements must be met. Also, the short half-life of the radioisotopes translates into short shelf life for the labeled reagents. Furthermore, those high-energy radioisotopes are attached to the binding molecule (e.g., antibodies) using specific chemistries that may modify its molecular behavior and interfere with the antigen−antibody interactions. These limitations of high-energy radioisotopes have prompted the development of other labeling and detection systems for clinical diagnosis-oriented immunoassays.

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Enzyme immunoassays (EIA) introduced in the early 1970s[6,7] and other EIA-based assays (e.g., fluorescent EIA and, later, chemiluminescent EIA) typically exhibit better analytical sensitivity than RIA/IRMA.[8,9] However, the sensitivity of these assays may be limited due to background native enzyme activities or fluorescent molecules present in almost all biological specimens. One solution to help eliminate background fluorescence is time-resolved fluorescence,[10] which has been successfully applied in an ultrasensitive prostate-specific antigen (PSA) assay.[11] Despite these advances, enzyme labeling is not always optimal for the detection of very low levels of peptides. Enzyme conjugation also involves coupling of one molecule to another. The coupling reactions may cause a change or reduction in immunological as well as biological activities of the labeled antibody or antigen. Due to these limitations, it is important to explore new labels and detection systems for peptide immunoassays.

A tumor marker is a substance produced by a tumor or by a host in response to the tumor's presence. Such substances can be measured in blood, fluid secretions, or tissues by immunoassays or by immunohistochemical and immunocytochemical stains. Tumor markers are used clinically for screening and early diagnosis of cancer in high-risk populations and for monitoring residual or recurrent malignant disease. As one example, serum PSA is routinely measured by immunoassays for the screening and diagnosis of prostate cancer in elderly male populations.[5,12,13] During the past few decades, tremendous resources have been devoted to identifying new tumor markers and developing sensitive and specific detection tools for them. Studies as early as the 1920s showed that at least 15−20% of malignant tumors (e.g., prostate cancer, breast cancer, squamous cell carcinoma of the lung, renal cell carcinoma, and adult T-cell leukemia/lymphoma) cause an increase in serum calcium levels without necessarily metastasizing to the skeleton.[14,15] It was not until the 1980s that parathyroid hormone-related protein (PTHrP) was discovered and later found to be the predominant cause for the humoral hypercalcemia of malignancy (HHM) by activating to the PTH/PTHrP receptor.[14−16] It has been shown that various cancers produce tumor-specific PTHrP fragments through alternative mRNA splicing and posttranslational processing.[17,18] Several secretory and circulating forms of PTHrP are being characterized.[19,20] Many of these PTHrP fragments have unique physiologic and pathophysiologic functions other than activating the PTH/PTHrP receptor.[16] Thus, PTHrP can serve as a useful tumor marker for a variety of cancers. However, the application of PTHrP as a tumor marker is hampered by the fact that currently used PTHrP immunoassays are not very sensitive or specific. In particular, no assays are available for the detection of various tumor-specific PTHrP isoforms.

Accelerator mass spectrometry (AMS) is a tandem mass spectrometry technique whose specificity and sensitivity can overcome the detection shortcomings of traditional immunoassay methods. AMS counts rare atoms by eliminating molecular isobars in high-energy collisions, followed by identification of individual ions by energy loss quantitation. AMS was developed in the late 1970s as a form of isotope ratio mass spectrometry for tracing long-lived radioisotopes (e.g., ^{14}C, half-life 5760 years) for chronometry in earth sciences and archaeological studies.[21] AMS can accurately measure $<10^6$ atoms (1 amol) of ^{14}C in a sample containing <1 mg of total carbon and can be used to analyze hundreds of samples per day. Unlike LSC, which counts decay events of a radioisotope, AMS is a mass spectrometric technique that detects individual ^{14}C nuclei. Over the past decade, AMS quantification of ^{3}H and ^{14}C has been applied to the life sciences in a variety of disciplines and has increased the precision and sensitivity of detection by several orders of magnitude.[22−28] Recently Shan et al. described an AMS-based homogeneous immunoassay for ^{14}C-labeled pesticides, in which the ^{14}C-labeled antigen was captured by a specific antibody and anti-immunoglobulin-coated magnetic beads, and the bound ^{14}C content on beads was further measured by AMS.[27]

Given the high specificity of two-site immunoassay and the attomole sensitivity of ^{14}C detection by AMS, we undertook the development of a sensitive and specific two-site immunoassay for a polypeptide using ^{14}C as the label and AMS as the detection system. One of the major PTHrP isoforms, PTHrP1−141 (MW ∼16 000), was selected as the prototypic analyte for the assay development. The feasibility and potential advantages of using ^{14}C as the label and AMS as the detection method in the two-site immunoassay format are presented in this article. Compared to conventional IRMA using high-energy and short-lived isotopes, the major advantage of our method is that this novel assay system is not only sensitive but also "nonradioactive", since the amount of ^{14}C used is far below the limit of disposal as radioactive waste. Although the nonspecific background remains a challenge in the lower analyte concentration range (<10 pmol/L), our results provide a foundation for further optimization of this potentially

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highly sensitive and laboratory environment-friendly immunoassay format. Based on the proposed assay format for PTHrP, similar assays can also be developed for other peptides, including hormones and other tumor markers. This report, to our best knowledge, represents the first description of a mass spectrometric two-site immunoassay for a peptide.

EXPERIMENTAL SECTION

Reagents. Human PTHrP1−34, PTHrP1−86, and PTHrP109−138 synthetic peptides were purchased from Bachem (Torrance, CA). Recombinant human PTHrP1−141 was generously provided by Drs. J. Moseley (University of Melbourne, Australia) and R. G. Hammonds (Genentech, South San Francisco, CA). The [U-^{14}C]-D-glucose and [U-^{14}C]L-leucine were purchased from ICN Biomedicals (Costa Mesa, CA). Na^{125}I was purchased from Amersham Biosciences (Piscataway, NJ). DMEM and RPMI 1640 media were purchased from Mediatech/Cellgro (Herndon, VA). The streptavidin−β-galactosidase and 4-methyl umbelliferyl−β-D-galactopyranoside (MUG) were purchased from Calbiochem (San Diego, CA). Other materials used include fetal bovine serum (FBS) from Omega Scientific (Tarzana, CA), bovine serum albumin (BSA) from US Biological (Swampscott, MA), protein assay reagents from Bio-Rad Laboratories (Hercules, CA), Ecoscint from National Diagnostics (Atlanta, GA), CNBr−Sepharose-4B resin from Sigma (St. Louis, MO), and protease inhibitors (phenymethylsulfonyl fluoride, aprotinin, leupeptin, pepstatin) from Sigma.

Monoclonal Antibodies for PTHrP. Monoclonal mouse antibodies against human PTHrP1−34 and PTHrP109−138 were generated by the standard hybridoma technology.[1] Briefly, the preparation steps include immunization of mice, collection of immune spleen cells, preparation of myeloma cells, fusion of myeloma cells with immune spleen cells, and cloning of hybridoma cell lines by limiting dilution. The cloned antibody producing hybridoma cell lines were propagated and frozen with dimethyl sulfoxide/fetal calf serum in liquid nitrogen for long-term preservation. A large quantity of monoclonal antibody was produced by growing the hybridoma cells in vitro or by inoculating hybridoma cells intraperitoneally into mice to generate ascites. The antibody was purified from hybridoma culture supernatant or hybridoma ascites fluid by protein G columns (HiTrap protein G 1-mL columns, Pharmacia Biotech, Piscataway, NJ). The reactivity and specificity of the purified antibodies, 1A5 (monoclonal mouse anti-human PTHrP1−34 antibody) and 8A8 (monoclonal mouse anti-human PTHrP109−138 antibody), were evaluated and confirmed by antigen binding assays and immunoaffinity purification efficiency.

Specificity and Binding Efficiency of the Monoclonal Antibodies. Peptides (PTHrP1−86 and PTHrP109−138) were labeled with ^{125}I by the Chloramine-T method and used for the antibody evaluation. For antigen binding assays, samples were first made by serial dilution (e.g., 1:10, 1:100, and 1:1000) of each original antibody eluate fraction. The assay was performed in 10 × 75 mm glass tubes by the addition of 100 μL of sample, 100 μL of ^{125}I-labeled tracer peptide (PTHrP1−86 or PTHrP109−138) (∼10 000 cpm), and 200 μL of diluent buffer (0.25% BSA in 0.2 M sodium phosphate, pH 7.5) to each tube, followed by overnight incubation at 4 °C. The antigen−antibody complex, if present, was precipitated by addition of 700 μL of secondary antibody solution (goat anti-mouse IgG) and incubation for 2−4 h at 4 °C. The tubes were then centrifuged at 2100 rpm and 4 °C for 1 h. Supernatants were decanted into a collection container for proper disposal, and the tubes containing the pellets were loaded into a γ counter and counted for 1 min.

For further specificity and binding efficiency evaluation, the antibody was coupled to cyanogen bromide activated Sepharose-4B resin (CNBr−Sephrose-4B resin). The prepared antibody-bound resin was equilibrated and kept at 4 °C in TBS (0.5 M NaCl, 20 mM Tris, pH 7.4) at a volume ratio of 1:1. At the time of evaluation, 200 μL of equilibrated beads/TBS (∼100 μL of beads) and 800 μL of DMEM containing ∼1 × 10^5 cpm of ^{125}I-labeled peptides (PTHrP1−86 or PTHrP109−138) were added to each microcentrifuge tube, followed by overnight incubation at 4 °C. The tubes were then spun for 1−2 min at 10 000 rpm, and the supernatant was collected for radioactivity counting. After washing the beads with TBS two times, 1.0 mL of elution buffer (0.5 N acetic acid) was added followed by 5-min incubation at room temperature. The tubes were spun again, and both the eluate and resin were collected for radioactivity counting. The supernatant, acid eluate, and beads were collected into 10 × 75 mm glass tubes, which were then loaded into a γ counter, and each tube was counted for 1 min.

Labeling of Monoclonal Antibody with ^{14}C. 1A5, which was used as the detection antibody in the two-site immunoassay, was labeled with ^{14}C by incubating the hybridoma cells (∼40 million cells) with culture media (400 mL of RPMI with 10% FBS) containing 250 μCi of [U-^{14}C]D-glucose and 250 μCi of [U-^{14}C]L-leucine. A miniPERM bioreactor (Vivascience, Hannover, Germany) was used for the high-density cultivation of hybridoma cells and for the labeling of secreted antibodies. The bioreactor has two modules, the production module and the nutrient module. When the two modules are attached together, a semipermeable membrane separates the two from each other. The production module has a volume of ∼40 mL and contains the hybridoma cells, and the nutrient module can hold up to 400 mL of culture media. The semipermeable membrane allows small molecules (e.g., nutrients, products of cell metabolism, and gases) to diffuse freely but prevents the secreted antibody from diffusing into the much larger nutrient module. Consequently, the antibody secreted by the hybridoma cells is highly concentrated in ∼40 mL of medium. During the cultivation, the bioreactor was rotated on a bottle-turning device in a 37 °C, CO_2 incubator at a relative humidity of ∼70%. After 3−4 days of cultivation, the medium containing the secreted antibody in the production chamber was collected for antibody purification.

Purification and Evaluation of the ^{14}C-Labeled Antibody. The ^{14}C-labeled antibody was purified and concentrated from the hybridoma culture media by affinity chromatography using the HiTrap protein G 1-mL columns (Pharmacia Biotech). Protein G, a cell surface protein of group G *Streptococci*, is a type III Fc receptor that binds to the Fc region of IgG by a nonimmune mechanism. The column was first washed/equilibrated with 5 mL of binding buffer (20 mM sodium phosphate, pH 7.0), followed by loading the hybridoma supernatant (premixed with the binding buffer at a volume ratio of 1:2) to the column by using a syringe. After washing away the unbound proteins and other constituents with 10 mL of binding buffer, the antibody was eluted from the column with the elution buffer (100 mM glycine hydrochloride,

pH 2.7). A total of 10 eluate fractions (~0.5 mL each) were collected to microcentrifuge tubes each containing 100 μL of neutralizing buffer (1.0 M Hepes, pH 8.0). The fractions were kept at 4 °C for the long-term preservation of antibody activity.

The 10 eluate fractions were evaluated for their protein concentration, radioactivity, and binding activity to PTHrP1−86. The protein concentration was determined by using the Bio-Rad DC protein assay reagents in a microtiter plate format according to the manufacturer's protocol. The radioactivity was determined by LSC using a β counter. An aliquot of 10 μL of each fraction was used for LSC. The binding activity and specificity were assessed by the aforementioned binding assays using ^{125}I-labeled PTHrP1−86 and PTHrP109−138.

Immunoassay Protocol. The test antigen used is recombinant human PTHrP1−141, one of the major PTHrP isoforms produced by many cancers. A pair of monoclonal antibodies, 8A8 (mouse anti-human PTHrP109−138 antibody) and ^{14}C-1A5 (^{14}C-labeled mouse anti-human PTHrP1−34 antibody), were used. The capturing antibody, 8A8 (1 μg/well), was first immobilized to 96-well microtiter plates (Immulon-4 HB, Dynex Technology, Chantilly, VA), followed by 2-h blocking at room temperature (or overnight blocking at 4 °C) with the blocking buffer (3% BSA in PBS). A series of PTHrP1−141 standard solutions (0, 32, 160, 800, 4000, 10 000, and 20 000 pg/mL) were prepared in diluent buffer (1% BSA in PBS or DMEM with 10% FBS; both were supplemented with 1.5 mM phenylmethylsulfonyl fluoride, 0.2 units/mL aprotinin, 1 μM leupeptin, 1 μM pepstatin, and 5 mM EDTA). The detection antibody, ^{14}C-1A5 (eluate fraction 4, see above), was diluted at 1:2500 in the diluent buffer. Each experiment was done in at least triplicate in order to generate a standard curve. A 100-μL aliquot of each analyte standard and a 50-μL aliquot of the diluted ^{14}C-1A5 were added sequentially to each well, followed by incubation with gentle shaking at room temperature for 2 h (or at 4 °C for 8−12 h). After decantation and the washing steps (washing buffer, 0.1% Tween-20 in PBS), a 100-μL aliquot of 1 N HCl was added to each individual well and the resultant mixture incubated for 10−20 min at room temperature to strip the [8A8-PTHrP1−141−^{14}C-1A5] complex off the well. Each sample was then carefully collected into separate microcentrifuge tubes, and the bound ^{14}C content was measured by using AMS.

The immunofluorometric immunoassay (IFMA) scheme is similar except for the labeling and detection methods. The detection antibody used was biotinylated 1A5, which was prepared by the *N*-hydrosuccinimide ester method. After incubation with the biotinylated 1A5 at 4 °C for 16−18 h, the detection was accomplished by the incubation with streptavidin-β-D-galactosidase for 1 h and signal development for 2−4 h with the fluorogenic substrate, 4-methyl umbelliferyl-β-D-galactopyranoside according to a sandwich ELISA protocol (BioSource International, Camarillo, CA). The plates were read in a fluorometric plate reader (Wallac, Gaithersburg, MD) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

AMS Sample Preparation and Measurement. The samples were individually mixed with 50 μL of carbon carrier solution (40 mg/mL tributyrin in methanol) before drying in a vacuum centrifuge. The samples were converted to graphite for AMS analysis with each containing ~1.2 mg of carrier carbon (tributyrin containing 8.8 amol of ^{14}C/mg C).[27,28] Three tributyrin carrier blanks were prepared with each set of fractions. AMS measurements were performed at the Lawrence Livermore National Laboratory. Typical AMS measurement speed was 3 min/sample, with a counting precision of 1.4−2.0% and a SD among three to seven measurements of 1−3%. The ^{14}C/^{13}C ratios of the unknowns were normalized-to-measured ratios of four identically prepared standards of known isotope concentration. The results were reported as modern carbon (naturally occurring environmental ^{14}C), which equals 97.9 amol (10^{-18} mol) of ^{14}C/mg of carbon.

Table 1. Binding Specificity and Efficiency of the Monoclonal Antibodies

antibody	^{125}I-labeled peptides	amount (%) in supernatant	amount (%) on Sepharose beads	amount (%) in eluate
1A5[a]	PTHrP1−86	30	70	60
	PTHrP109−138	98	2	0
8A8[b]	PTHrP1−86	95	5	0
	PTHrP109−138	22	78	67

[a] Anti-PTHrP1−34 antibody. [b] Anti-PTHrP109−138 antibody.

RESULTS AND DISCUSSION

Specificity and Binding Efficiency of the Monoclonal Antibodies. The specificity and binding efficiency of the generated monoclonal antibodies were evaluated by both antigen binding assay and immunoaffinity efficiency assessment. As mentioned, 1A5 and 8A8 are the monoclonal antibodies against human PTHrP1−34 and PTHrP109−138, respectively. In the binding assays, 1A5 at up to 1:5000 dilution exhibits specific binding activity only to ^{125}I-PTHrP1−86 and ^{125}I-PTHrP1−34 but not ^{125}I-PTHrP109−138. On the other hand, 8A8 at up to 1:10 000 dilution exhibits specific binding activity only to ^{125}I-PTHrP109−138, but not to ^{125}I-PTHrP1−34 or ^{125}I-PTHrP1−86 (data not shown). As shown in Table 1, the immunoaffinity efficiency assessments not only further confirmed the specificity of 1A5 and 8A8 but also showed that both antibodies can be used for immunoaffinity purification for relevant PTHrP fragments with high efficiency.

Labeling of the Capturing Antibody (1A5) with ^{14}C. To ensure 1A5 was successfully labeled with ^{14}C, we evaluated the 10 eluate fractions from the protein G affinity column for its protein concentration (μg/mL), radioactivity (nCi/mL), and binding activity to ^{125}I-PTHrP1−86. We then correlated the radioactivity and protein concentration with the binding activity. As shown in Table 2, both the radioactivity and protein concentration correlated with the binding activity ($R = 0.92$, respectively) with fraction 4 having the highest activities. The results indicate that the 1A5 eluted from the protein G column was pure, functional, and adequately labeled with ^{14}C. Because fraction 4 has the highest concentration of ^{14}C-1A5, we chose to use this fraction in the AMS-based two-site immunoassay development.

Immunofluorometric Assay for PTHrP1−141. Before studying the mass spectrometric immunoassay, we first tried IFMA to determine the optimal assay conditions, including the blocking and diluent buffers and the dynamic range. A pair of antibodies was used with 8A8 as the capturing antibody and biotinylated 1A5 as the detection antibody. Based on the results

Table 2. Evaluation of the ^{14}C-1A5 Eluate Fractions from the Affinity Chromatography

eluate fraction	1	2	3	4	5	6	7	8	9	10
protein (μg/mL)	21	25	69	201	110	66	44	37	37	31
radioactivity (^{14}C, nCi/mL)	29	37	243	837	455	278	162	135	134	120
binding activity (^{125}I, cpm)	0	0	142	253	171	151	62	48	74	0

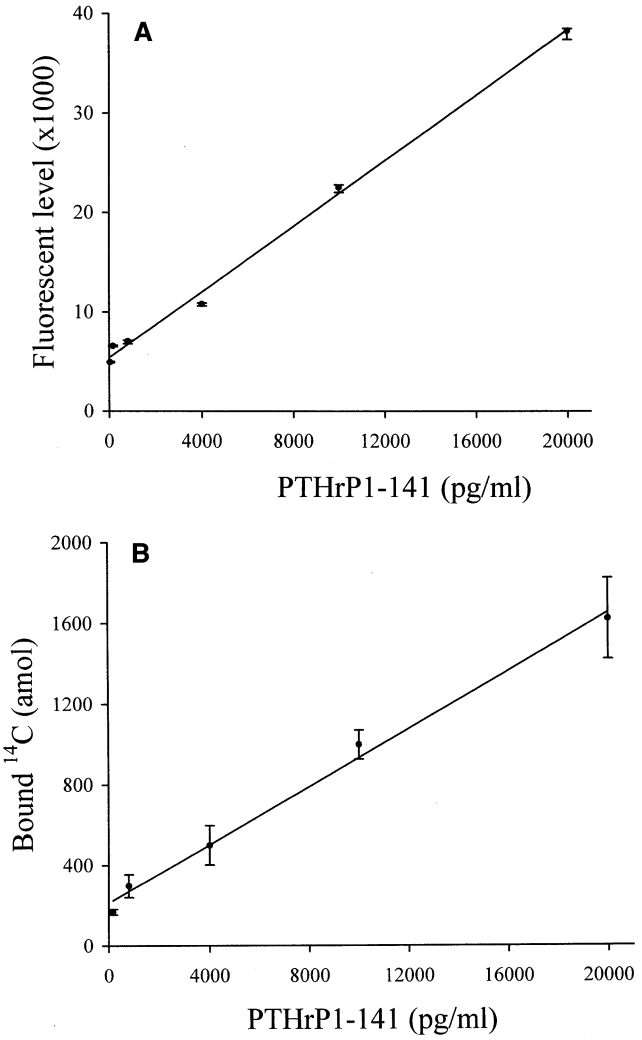

Figure 1. PTHrP standard curves. (A) Typical standard curve obtained with the IFMA for PTHrP1−141. Data are presented as means ± SD of triplicate measurements and are represented directly by the fluorescence levels (count, ×1000). Shown here is from 32 to 20 000 pg/mL PTHrP1−141. (B) Typical standard curve obtained with the mass spectrometric two-site immunoassay for PTHrP1−141. Data are expressed as means ± SD of triplicate measurements and are represented directly by the detected bound ^{14}C levels (attomole). Shown here is from 160 to 20 000 pg/mL PTHrP1−141.

from several preliminary experiments, it was determined that the nonspecific background was the lowest by using 3% BSA in PBS (phosphate buffer solution) as the blocking buffer and 10% FBS in DMEM or 1% FBS in PBS as the diluent buffer (data not shown). Figure 1A shows a typical PTHrP1−141 IFMA standard curve when 10% FBS in DMEM was used as the diluent. The analytical sensitivity (or limit of detection) was determined to be 32 pg/mL (2 pmol/L) with a linear measurement range up to 20 000 pg/mL (∼1.3 nmol/L).

The assay specificity was examined by using the same scheme for the measurement of a serial standard dilutions of PTHrP1−86 and PTHrP109−138 in the diluent buffer (10% FBS in DMEM). The same pair of capturing and detection antibodies was used. As expected, both peptides generated flat curves with the fluorescence levels close to the blank controls (data not shown).

For interference assessment, PTHrP1−86 (4000 pg/mL) was spiked into the PTHrP1−141 standards. The presence of PTHrP1−86 did not interfere the PTHrP1−141 assay, and the resulting standard curve was the same as that without PTHrP1−86 in the standards (data not shown). The results indicated that the IFMA using 8A8 and 1A5 only detects PTHrP1−141 without cross-reaction to other PTHrP fragments.

Mass Spectrometric Immunoassay for PTHrP1−141. Based on the results of the IFMA, we chose to use 3% BSA in PBS as the blocking buffer and 1% BSA in PBS as the diluent buffer. The PTHrP1−141 standards used were the same as those used for the IFMA, including 0, 32, 160, 800, 4000, 10 000, and 20 000 pg/mL solutions. The affinity-purified antibodies used in the assay ensured the strong immunoreaction of antigen−antibody binding and relatively low background of ^{14}C. Figure 1B shows a typical PTHrP1−141 standard curve for the ^{14}C-AMS based immunoassay. The background ^{14}C level was 137± 22 amol (mean ± SD), which could be distinguished from that of 160 pg/mL standard ($p < 0.05$). No high-dose "hook" effect was observed at the highest concentration point of 20 000 pg/mL. Based on three separate experiments, the analytical sensitivity was determined to be 160 pg/mL (10 pmol/L) with a linear dynamic range up to 20 000 pg/mL (∼1.3 nmol/L). These result are similar to those obtained with the IFMA.

Control wells were included to ensure there was no significant nonspecific binding to the inner surface of the microtiter wells by PTHrP1−141 itself. The control wells were not coated with the capturing antibody 8A8; otherwise, they are treated the same way as the other experimental wells. Without 8A8 coating, the levels of ^{14}C detected were very low, even in those containing 20 000 pg/mL standard, with an average ^{14}C level of only 35 amol/well. The results indicated that there was no significant nonspecific binding of antigen to the inner surface of the wells. However, coating with 8A8 led to a significant increase in the background noise with an average ^{14}C level of 137 amol in the blanks.

The two-site assay utilizes a stoichiometric excess of reagent antibodies. When background noise is omitted, the ultimate sensitivity of the sandwich assay is one molecule of analyte, which is theoretically possible when the amount of antibody used in the assay system approaches infinity. Also, the assay protocol can be simplified and a one-step sandwich assay can be performed. In this study, the immobilized capturing antibody, the antigen PTHrP1−141, and the ^{14}C-labeled detection antibody were mixed together simultaneously. Interference did not occur because the

pair of antibodies recognized different antigenic sites. The signal generated was proportional to the amount of PTHrP added to the microtiter wells. In sandwich assays, the sensitivity is determined by the association constant (affinity or avidity) of the antigen−antibody interaction, the signal intensity of the labels, and the signal-to-noise ratio. Since the antibodies (detection antibody 1A5, capturing antibody 8A8), the antigen (PTHrP1−141), and the ^{14}C-labeling are fixed, it is necessary to reduce the background noise to increase the analytical sensitivity. At this time, the background noise (an average bound ^{14}C level of 137 amol/well) is notably high given the average ^{14}C level in the carrier blank samples was only ∼10 amol. The background noise appears to relate to the presence of the capturing antibody 8A8 because the average ^{14}C level detected in 8A8-negative control wells was only 35 amol. It is not clear whether the noise is caused by 8A8 itself or by nonspecific interactions between 8A8 and PTHrP1−141 or ^{14}C-1A5. Obviously the main challenge is to further diminish the background noise in order to obtain higher analytical sensitivity.

To document the correlation and difference between this novel assay and conventional RIA or IFMA when real-life biological samples (e.g., culture media, serum, etc.) are used, we measured PTHrP in conditioned culture media collected from wild-type COS-1, a African green monkey kidney cell line, which secretes undetectable amounts of PTHrP, and a stable PTHrP1−173 genetically transformed COS-1 clone, which secrets abundant PTHrP as measured by RIA, the "gold-standard" PTHrP assay used in our laboratory. PTHrP1−141 was undetectable in the media from the wild-type cells; however, 5100 pg/mL PTHrP1−141 was present in the media from the transformed cells as determined by the AMS-based sandwich assay. The results was similar to that generated from the RIA and IFMA.

It was assumed that the assay specificity is similar to the IFMA. The two assays are basically the same except for different labeling (^{14}C-labeling vs biotinylation) and detection methods (AMS vs fluorescence reader) as described in the Experimental Section.

CONCLUSION

In an effort to explore new labeling and detection system for two-site or sandwich immunoassay for peptides, we have demonstrated that monoclonal antibodies can be easily and efficiently labeled with ^{14}C and that AMS can be applied to peptide immunoassays. The ^{14}C-AMS-based PTHrP1−141 sandwich immunoassays we developed are comparable to the PTHrP IFMA in terms of analytical sensitivity and linear dynamic range. Although the nonspecific background remains a challenge and the sensitivity is still far from that theoretically achievable by AMS, this work can serve as a foundation for further optimization and improvement of this novel peptide sandwich assay format. This work also represents another step closer to the ultimate goal of bringing the highly sensitive AMS detection technology to clinical laboratories.

Compared to other labels used in immunoassays, ^{14}C-labeling is obviously advantageous for a number of reasons. First, carbon is ubiquitous and rich in almost all chemicals including peptides. Carbon isotope ^{14}C can be easily incorporated into the antibody or antigen during the biological or chemical synthetic steps and therefore no extra coupling or conjugation procedure is necessary. It was demonstrated in the present study that monoclonal antibody can be efficiently labeled when [U-^{14}C]D-glucose and [U-^{14}C]L-leucine were added to the media used for the high-density cultivation of hybridoma cells. Both glucose and leucine are required and used by the cells while producing and secreting specific antibodies. Similarly, recombinant protein produced by bioengineering technology can be efficiently labeled with ^{14}C if [U-^{14}C]D-glucose is added to the bacterial culture media.[25] Therefore, both antibody and peptide antigen can be labeled with ^{14}C and produced in large quantities by using the currently available technologies. Second, ^{14}C has a half-life of 5760 years and thus the labeled reagents should be valid and ready to use as long as the reagent retains its biological activities. That characteristic can overcome the problem of short shelf life of labeled reagents when short-life radioisotopes are used. Third, ^{14}C can be detected at attomole (10^{-18} mol) level by AMS, and therefore, the amount of ^{14}C required in the immunoassay is well below the designated limit (>50 nCi/g) of disposal as radioactive waste. By using ^{14}C as the label and AMS as the detection system, the immunoassay can be practically considered as "nonradioactive" with no radiation safety concerns in a clinical laboratory environment. For example, in our study, the amount of ^{14}C used is only 16.7 pCi/well or 1.6 nCi/96-well microtiter plate, which is far below the limit to be considered radioactive waste.

The access to AMS is currently limited due to the relatively few numbers of instruments available for biological studies. In addition, current AMS requires preparation of solid graphite samples for measurement that could limit throughput for high-volume and complex analyses. However, a variety of biological and biomedical applications[18−25] are driving the development of accelerator mass spectrometers (for isotopes such as ^{3}H and ^{14}C) that are small, automated, of reduced cost, and of high throughput. Due to its attomole sensitivity for signal detection, AMS use in biological sciences, including clinical diagnosis and patient care, will increase dramatically soon.

Future directions of this methodology include further optimization of the mass spectrometric immunoassay, identification of tumor-specific PTHrP isoforms, generation of specific antibodies of high affinity and development of sensitive mass spectrometric immunoassays for those isoforms, and ultimately clinical application of the assays for the screening and early diagnosis of primary, residual, or recurrent PTHrP-expressing cancers.

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