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Enhanced Target-Specific Accumulation of Radiolabeled Antibodies by Conjugating Arginine-Rich Peptides as Anchoring Molecules

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We have devised and estimated a new strategy to prolong the residence time of radiolabeled antibodies in tumor in which an octaarginine peptide (R_8) was used as an anchoring molecule to fix antibodies against CD20 (NuB2; IgG2a) on tumor cells. Conjugation of R_8 with antibodies was performed by maleimide−thiol chemistry using thiol groups generated by reducing the disulfide bonds of the antibody. The R_8-conjugated NuB2 was then reacted with succinimidyl *meta*-[^{125}I]iodobenzoate to prepare [^{125}I]SIB-NuB2$_I$ (0.92 R_8/NuB2) and [^{125}I]SIB-NuB2$_{III}$ (3.38 R_8/NuB2). Both SIB-NuB2$_I$ and SIB-NuB2$_{III}$ exhibited size-exclusion HPLC elution profiles and immunoreactivity to CD20-positive cells similar to those of NuB2. NuB2$_I$ also possessed isoelectric focusing (IEF) profile similar to NuB2. However, NuB2$_{III}$ registered a broad IEF band toward higher pI. When incubated with CD20-positive cells, [^{125}I]SIB-NuB2$_I$ and [^{125}I]SIB-NuB2$_{III}$ exhibited 1.4 and 4.0 times higher cell-associated radioactivity than [^{125}I]SIB-NuB2. After the cells were washed and reincubated in a fresh medium for 3 h, [^{125}I]SIB-NuB2$_I$ and [^{125}I]SIB-NuB2$_{III}$ exhibited significantly higher cell-associated radioactivity than [^{125}I]SIB-NuB2. In biodistribution studies in normal mice, while both [^{125}I]SIB-NuB2$_I$ and [^{125}I]SIB-NuB2 exhibited similar biodistribution profiles, [^{125}I]SIB-NuB2$_{III}$ showed faster clearance from the blood and higher hepatic radioactivity levels than [^{125}I]SIB-NuB2. In SCID mice bearing CD20-positive xenografts, [^{131}I]SIB-NuB2$_I$ exhibited significantly higher radioactivity in xenografts than those of [^{125}I]SIB-NuB2 with no significant increase being observed in other tissues. The findings indicate that appropriate R_8 modification of antibodies satisfies both specific targeting ability of antibody and strong cell-association property of R_8, which was reflected in the increased radioactivity levels in tumor. These findings supported the applicability of this approach to enhance target-specific accumulation of radiolabeled antibodies.

INTRODUCTION

Radioimmunotherapy (RIT) is a cancer therapeutic modality that combines the specific tumor targeting of immunotherapeutics and cytotoxic radiation mechanisms. Encouraging results have been reported in patients with hematologic malignancies treated with ^{90}Y- and ^{131}I-labeled antibodies against CD20 (*1*−*4*), due to their intrinsic high radiosensitivity and relatively good access of the radiolabeled antibodies to the cancer cells (*5, 6*). Despite numerous efforts, the treatment of solid tumors with radiolabeled antibodies resulted in limited success, due to poor localization of radiolabeled antibodies in the tumors (*7*−*9*). Thus, the enhancement of tumor accumulation and retention of radiolabeled antibodies constitutes a prime requisite for successful RIT.

Numerous factors have been presented as being responsible for tumor accumulation and retention of radiolabeled antibodies (*8*). Since the mechanism of accumulation of radiolabeled antibodies is based on their specific but reversible binding to antigens in tumor tissues, a strategy that shifts the equilibrium to antigen association was considered of prime importance to enhancing tumor accumulation. The use of antibodies internalized to target cells would prolong residence time in the tumor, since these molecules will be resistant to being cleared by washout. The conjugation of arginine-rich cell-penetrating peptides (AR-CPPs) to antibodies has been conducted as a means to facilitate internalization of antibodies and to increase tumor accumulation. Indeed, a prior study reported an increase in tumor accumulation of AR-CPP-conjugated antibodies in vitro (*10*). However, in vivo studies showed increased accumulation in nontarget tissues such as the liver with decreased accumulation in tumors. A prior study also described that the tumor targeting ability of an antibody construct (scFv) was completely abolished following conjugation with AR-CPP (*11*).

Meanwhile, an increase in avidity of antibodies constitutes another strategy to enhance tumor accumulation, as observed in antibody constructs with multivalent binding domains (*8, 12, 13*). An increase in tumor uptake was also observed with divalent radiolabeled haptens in pretargeting strategy (*14*). These studies suggested that chemical modification of antibodies with anchoring molecules that possess strong interaction with cell surface molecules or cell membrane may also be useful to shift the equilibrium to antigen association in tumor tissues.

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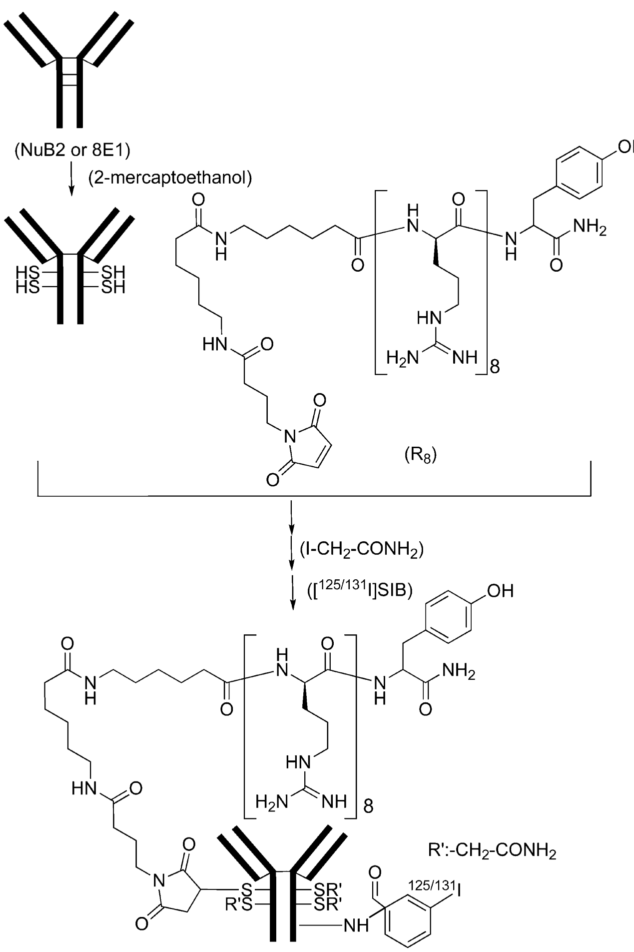
Scheme 1



The AR-CPPs possess strong interaction with phosphate groups, sulfates, and carboxylates on cellular components (*15*−*18*). The electrostatic interaction between AR-CPPs and cell membrane is so strong that complete removal of AR-CPPs from the cell surface is difficult to perform (*19*−*21*). Such characteristics render AR-CPPs applicable as an anchoring molecule to shift the equilibrium to antigen association for prolonged retention of radiolabeled antibodies in tumors. In addition, the strong electrostatic interaction between AR-CPPs and cellular components suggested that AR-CPPs would exert their anchoring ability at conjugation levels much lower than those used for facilitating internalization.

In this study, an octaariginine peptide was selected as an anchoring molecule, and the peptide was derivatized with a maleimide group to conjugate with a relevant or irrelevant antibody against CD20 (Scheme 1). After radiolabeling of the modified antibodies with succinimidyl *meta*-[$^{125/131}$I]iodobenzoate ([$^{125/131}$I]SIB), the effect of the octaarginine conjugation on specific binding to target cells, dissociation rates from the cells, and biodistribution was determined with special emphasis being laid on the relationship between the anchoring ability of R_8 and the number of R_8 molecules attached per molecule of the antibody. The applicability of the new approach using an AR-CPP as an anchoring molecule to prolong residence time of radiolabeled antibodies in tumor will be discussed.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Na[^{125}I]I and Na[^{131}I]I were obtained from MP Biomedicals (Irvine) and Perkin-Elmer (Yokohama, Japan), respectively. The stannyl precursor of the radioiodination reagents, *N*-succinimidyl-3-(tri-*n*-butylstannyl)-benzoate (ATE) was synthesized as reported previously (*22*). Size-exclusion high-performance liquid chromatography (SE-HPLC) was performed using a Cosmosil Diol-300 (7.5 × 600 mm, Nacalai Tesque, Kyoto, Japan) column at a flow rate of 1 mL/min with 0.1 M phosphate buffer (PB, pH 6.8) as an eluent. Each eluent was collected with a fraction collector (RediFlac, GE Healthcare Bioscience, Tokyo, Japan) at 1 min intervals. The radioactivity counts in each fraction (1 mL) were determined with an autowell γ counter (ARC-380M, Aloka, Tokyo). TLC analyses were performed with silica plates (Silica Gel 60 F_{254}, Merck, Tokyo). Isoelectric focusing (IEF) was conducted using a Tefco STC808 mini electrophoresis cell (Tefco, Tokyo) with a precast polyacrylamide gel isoelectric focusing (IEF-PAGE) mini isocratic gel (pH 3−10) (Tefco, Tokyo). Other reagents were of reagent grade and were used as received.

Cells and Antibodies. The CD20 positive human peripheral lymphocyte line RPMI 1788 cells were incubated with RPMI-1640 medium (Sigma-Aldrich Co, St. Louis, MO, USA) supplemented with 10% fetal calf serum and were used for the studies.

The monoclonal antibodies used in this study were a murine monoclonal antibody (NuB2; IgG2b) that reacts specifically with CD20 as a relevant antibody and a murine monoclonal antibody (8E1; IgG2a) specific to a neural protein as an irrelevant one, respectively, since both subclasses possess similar quaternary structures and the interchain disulfide bonds are the primary sites of chemical reduction for both antibodies (*23*). Both antibodies were kindly supplied from Immuno-Biological Laboratories Co, Ltd., (Takasaki, Japan).

Synthesis of Mal-(Acp)$_2$-(D-Arg)$_8$-(D-Tyr) (R$_8$). We employed D-octaarginine peptide as an AR-CPP, and the peptide with N-terminal bis-aminocaproate linker was coupled with *N*-(4-maleimidebutyryloxy)succinimide for antibody conjugation (Scheme 1).

The peptide chain of R_8 was constructed by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis on a TGS-RAM resin (Shimadzu, Kyoto) using Shimadzu PSSM8 peptide synthesizer (Shimadzu, Kyoto) with its standard protocol. A 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate/1-hydroxybenzotriazole/*N*,*N*-diisopropylethylamine coupling system was employed as the coupling system. As amino acid derivatives, Fmoc-Acp, Fmoc-D-Arg(Pdf), and Fmoc-D-Tyr(^{t}Bu) were used (Acp = 6-aminocaproic acid). The peptide resin was then treated with *N*-(4-maleimidobutyryloxy)succinimide (3 equiv) and *N*-methylmorpholine (3 equiv) in DMF at 37 °C for 20 min to yield a peptide resin bearing a maleimide moiety on its N-terminus. The final deprotection of the peptide resin using trifluoroacetic acid (TFA)−H_2O (95:5) at room temperature for 3 h followed by reverse-phase HPLC purification with a Cosmosil Protein-R (10 × 250 mm, Nacalai Tesque, Kyoto) at a flow rate of 1 mL/min with a gradient mobile phase starting from 14% A (H_2O containing 0.1% TFA) and 86% B (CH_3CN containing 0.1% TFA) to 44% A and 56% B in 15 min. The product was ascertained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS, Voyager-DE STR, Applied Biosystems, Tokyo). MALDI-TOFMS: 1822.14 [Calcd for $(M + H)^+$: 1822.16].

Preparation of R$_8$-Modified Antibodies. Two R_8-modified NuB2s with different R_8-conjugation levels ($NuB2_I$ and $NuB2_{III}$) were prepared by reducing the disulfide bonds of NuB2 as reported previously with some modifications (Scheme 1) (*22*). Briefly, NuB2 was concentrated in a nitrogen atmosphere to 6.0 mg/mL in well-degassed 0.1 M PB (pH 7.0) containing 2 mM EDTA. The antibody (1.0 mL) was allowed to react with 2-mercaptoethanol (2-ME, 1000 molar excess) by gently stirring at room temperature for 30 min. Excess 2-ME was then removed by a centrifuged column procedure using Sephadex G-50 Fine (GE Healthcare, Tokyo) equilibrated and eluted with 0.1 M PB (pH 6.0) containing 2 mM EDTA. A small aliquot of the filtrate was sampled, and the number of exposed thiol groups was determined with 2,2-dipyridyl disulfide (*24*). The filtrate was then added to a reaction vial containing 1.5-fold R_8 (42.8 μL, 85.7 μg) for NuB2$_I$ or 5-fold R_8 for NuB2$_{III}$ in 0.1 M PB (pH 6.0). After the reaction mixture was agitated gently for 1.5 h at 25 °C, 500-fold molar excess of iodoacetamide (269 μL, 2.68 mg) in 0.1 M PB (pH 6.0) was added. The reaction mixture was further incubated for 30 min to alkylate the unreacted thiol groups. NuB2$_I$ and NuB2$_{III}$ were finally purified by the centrifuged column procedure, equilibrated, and eluted with PBS (0.01 M, pH 7.4; Wako, Tokyo). Both 8E1$_I$ and 8E1$_{III}$ were prepared according to the procedure described above except for using 8E1 in place of NuB2.

Preparation of [$^{125/131}$I] *N*-Succinimidyl 3-Iodobenzoate (SIB)-Labeled Antibodies. *N*-Succinimidyl 3-(tri-*n*-butylstannyl)benzoate (ATE) was synthesized and radio-iodinated in the presence of *N*-chlorosuccinimide (NCS) as described previously (*25*). Briefly, ATE was dissolved in methanol containing 1% acetic acid (0.45 mg/mL), and 16.2 μL of this solution was mixed with 4.4 μL of NCS in methanol (0.5 mg/mL) in a sealed vial, followed by addition of Na[^{125}I]I (2 μL). After incubation at room temperature for 45 min, the reaction was quenched with aqueous sodium bisulfite (2.2 μL, 0.72 mg/mL). The radiochemical yield of [^{125}I]SIB was determined by TLC developed with ethyl acetate. The solvent was removed under a stream of N_2 prior to subsequent conjugation reaction with the antibodies. Conjugation of [^{125}I]SIB with antibodies was performed according to our previous procedure (*22*), with slight modifications as follows: A solution of NuB2$_I$, NuB2$_{III}$, 8E1$_I$, or 8E1$_{III}$ (120 μL, 2.5 mg/mL) in 0.2 M borate buffer (pH 8.5) was added to the dried residue of crude [^{125}I]SIB. After gentle incubation for 1 h at room temperature, [^{125}I]SIB-labeled antibodies were purified by the centrifuged column procedure, equilibrated, and eluted with PBS. Radioiodination of ATE with Na[^{131}I]I was performed as described above except for using Na[^{125}I]I in place of Na[^{131}I]I. Radiochemical purities of radiolabeled antibodies were also determined by SE-HPLC and TLC developed with 80% methanol/H_2O.

Preparation of ^{125}I-NuB2. Direct radioiodination of NuB2 was performed by the chloramine T method (*22*). To a solution of NuB2 (0.25 mg/mL; 200 μL) in 0.3 M PB (pH 7.5) was added 5 μL of Na[^{125}I]I. Chloramine T (0.1 mg/mL, 25 μL), freshly prepared in the same buffer, was then added. After incubation of the mixture for 10 min at room temperature, the reaction was terminated by an addition of 6 μL of aqueous sodium bisulfite (0.7 mg/mL). ^{125}I-NuB2 was purified by the centrifuged column procedure. The radiochemical purity was determined by SE-HPLC and TLC developed with 80% methanol/H_2O.

Preparation of Fluoresced Antibodies. NuB2, NuB2$_I$, and NuB2$_{III}$ were also labeled with Alexa Fluor 488 (AF, Molecular Probes, Tokyo). The fluorophore was dissolved in dimethylformamide at a concentration of 10 mg/mL, and the conjugation reaction was performed with a 5-fold molar excess of the fluorophore in 0.1 M sodium carbonate−sodium bicarbonate buffer (pH 9.0). After 1 h at room temperature, the reaction was terminated by an addition of freshly prepared 1.5 M hydroxylamine, and the fluorescent antibodies were purified by the centrifuged column procedure. The protein concentration in the purified conjugates was determined spectrophotometrically by subtracting 0.11 $A_{495\ nm}$ of the fluorophore from the $A_{280\ nm}$ of the antibody. The degree of labeling was calculated by dividing the $A_{495\ nm}$ of the fluorophore by the molar concentration of the antibodies and the dye extinction coefficient of 71 000 M^{-1} cm^{-1}.

Immunoreactivity. The immunoreactivity of radiolabeled NuB2s was assessed by the competitive immunoassay (*26*). To minimize nonspecific binding of radioiodinated antibodies to plastic tubes, the tubes were presaturated with a freshly prepared solution of 1% bovine serum albumin in PBS for 30 min, followed by three washes with PBS (*27*). Nonradioactive SIB-NuB2, SIB-NuB2$_I$, and SIB-NuB2$_{III}$ were prepared by experimental procedures similar to those used for [^{125}I]SIB-labeled antibodies, except that nonradioactive NaI was used in place of Na[^{125}I]I. A mixed solution of 50 μL ^{125}I-NuB2 and 50 μL nonradioactive SIB-NuB2, SIB-NuB2$_I$ or SIB-NuB2$_{III}$ (0.05−10 μg) was incubated in the presence of 1 × 10^6 RPMI 1788 cells suspended in 100 μL of PBS at 37 °C for 1 h. After centrifugation at 10 000 × *g* for 5 min, supernatant was discarded and the cell-associated radioactivity was determined using an auto γ counter.

RPMI 1788 Cell Binding and Retention. The cell binding of [^{125}I]SIB-labeled antibodies was also evaluated according to the procedure of Foulon et al. with slight modification (*28*). Approximately 1 μg each of [^{125}I]SIB-labeled antibodies was incubated with 1 × 10^6 RPMI 1788 cells for 1 h at 37 °C. Aliquots of cells in triplicate were removed, centrifuged, and washed with cold media. Then, the cell-associated radioactivity was determined as described above. Similar studies were conducted in the presence of 1000-fold excess of unlabeled NuB2.

The rest of the cells were resuspended in RPMI 1640 media containing 10% fetal bovine serum and 20 mM HEPES to a density of 3 × 10^6 cells/mL. After incubation for 1 and 3 h, aliquots of cells in triplicate were removed, centrifuged, and washed twice with cold media, and the cell-associated radioactivity was determined.

Confocal Microscopy. For each assay, 6 × 10^6 cells were plated into 35 mm glass-bottomed dishes. The cells were then incubated at 37 °C for 1 h with a fresh solution of NuB2, NuB2$_I$, or NuB2$_{III}$ (60 μL, 0.1 mg/mL) in cold RPMI 1640 media containing 10% FCS and 20 mM HEPES. The cells were washed twice with the same medium at 4 °C. The acidotropic dye LysoTracker Red DND-99 (Molecular Probes, Eugene, OR) was diluted in DMSO. The cells were resuspended in prewarmed (37 °C) medium containing 50 nM LysoTracker Red for 30 min. For live-cell imaging, aliquots of cells in 35 mm glass-based dishes were analyzed by confocal microscopy using a Fluoview FV500 (Olympus, Tokyo). Images were obtained at low-power laser (0.3−3.0% laser) using the 488 nm line of an argon laser. All images were obtained as 10 μm thicknesses of one planar (*xy*) section. The cells were maintained at 37 °C in a temperature-controlled box.

In Vivo Studies. Animal studies were conducted in accordance with our institutional guidelines and were approved by Chiba University Animal Care Committee. Biodistribution studies were performed by intravenous administration of a PBS solution of [^{125}I]SIB-NuB2, [^{125}I]SIB-NuB2$_I$, or [^{125}I]SIB-NuB2$_{III}$ to 6-week-old male ddY mice (Japan SLC Inc., Shizuoka, Japan). Groups of five mice, each receiving 0.3 μCi (20 μg/100 μL) of the antibodies, were used for the experiments. Organs of interest were removed and weighed, and the radioactivity counts were determined with an autowell γ counter at 1, 3, and 24 h postinjection.

Severe combined immunodeficiency (SCID) mice (C.B-17/IcrCrj-scid, Charles River Japan, Kanagawa, Japan) bearing xenograft of RPMI 1788 cells were also treated with a mixed solution of [^{131}I]SIB-NuB2$_I$ and [^{125}I]SIB-NuB2. The biodistribution of radioactivity after i.v. administration of the mixture

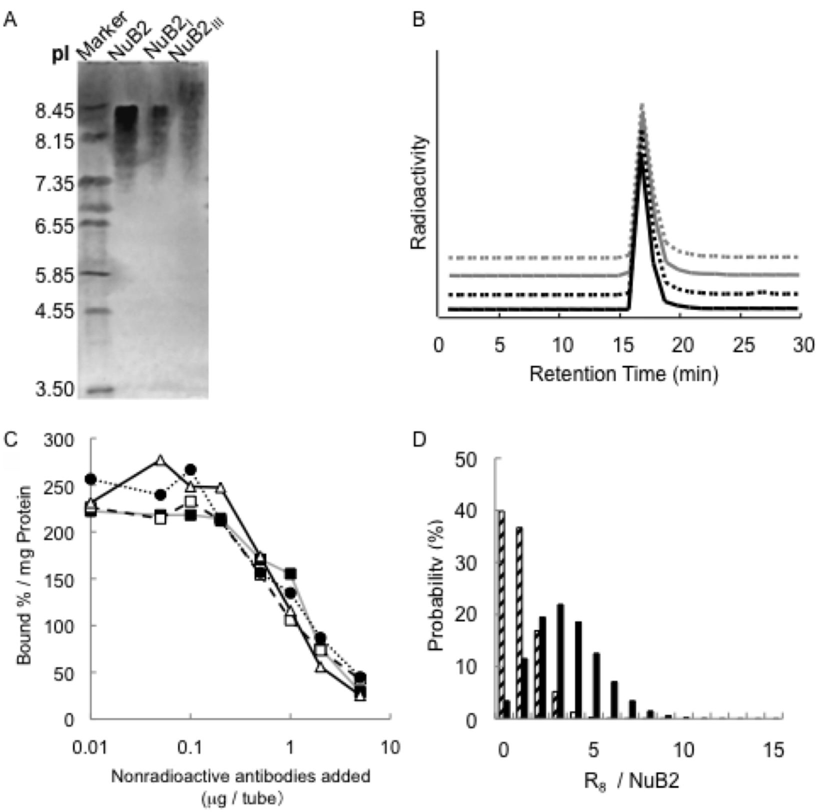


Figure 1. Characterization of R_8-conjugated antibodies ($NuB2_I$ and $NuB2_{III}$). (A) Isoelectric focusing. While both NuB2 and $NuB2_I$ showed similar profiles, a wider band toward higher pI was observed with $NuB2_{III}$. (B) Size-exclusion HPLC profiles. ^{125}I-NuB2: black solid line. [^{125}I]SIB-NuB2: black broken line. [^{125}I]SIB-$NuB2_I$: gray solid line. [^{125}I]SIB-$NuB2_{III}$: gray broken line. All the radioiodinated NuB2s exhibited similar elution profiles. (C) Competitive immunoassay to RPMI 1788 cells. Increasing concentrations of nonradioactive iodine-labeled NuB2s (NuB2 (solid square), SIB-NuB2 (square), SIB-$NuB2_I$ (circle), SIB-$NuB2_{III}$ (triangle)) and 1×10^6 RPMI 1788 cells were incubated in the presence of [^{125}I]-labeled NuB2. No significant differences were observed among the four NuB2s. (D) Poisson distribution profiles of $NuB2_I$ (hatched column) and $NuB2_{III}$ (solid column).

to 8-week-old SCID mice was monitored at 24, 48, 72, and 96 h postinjection. Groups of 5−7 mice, each receiving 0.3 μCi (10 μg each) of antibodies, were used for the experiments. Organs of interest were removed and weighed, and the radioactivity counts were determined with an autowell γ counter. A window from 29 to 97 keV was used for counting ^{125}I, whereas one from 280 to 440 keV was used for ^{131}I. Correlation factors to eliminate crossover of ^{131}I activity into ^{125}I were determined by counting ^{131}I standard in each window. The crossover of ^{125}I into the ^{131}I channel was negligible.

Statistical Analysis. Data are expressed as means ± standard deviation where appropriate. Results were statistically analyzed with an unpaired Student's *t*-test using the Microsoft *Excel* program. Differences were considered statistically significant when the p value was less than 0.05.

RESULTS

Preparation of Conjugates. Conjugation of NuB2 or 8E1 with R_8 was performed by maleimide−thiol chemistry using thiol groups generated by reducing the disulfide bonds of the antibody molecule. The numbers of R_8 molecule attached per molecule of NuB2 were determined to be 0.92 for $NuB2_I$ and 3.38 for $NuB2_{III}$. Similar procedures were employed for the preparation of $8E1_I$ and $8E1_{III}$ where the number of R_8 per molecule of 8E1 was estimated to be 0.98 and 3.85, respectively. The pI values of NuB2, $NuB2_I$, and $NuB2_{III}$ were determined by the isoelectric focusing and found to be 7.5−8.6, 7.5−8.6, and 7.5−9.0, respectively (Figure 1A). All [$^{125/131}$I]SIB-labeled antibodies had radiochemical purities over 95% when determined by the SE-HPLC (Figure 1B) and TLC. ^{125}I-NuB2 was prepared by the chloramine T method with radiochemical purity of 99%. AF-NuB2, AF-$NuB2_I$, and AF-$NuB2_{III}$ were labeled with Alexa Fluor 488 with a conjugation level of 3.67, 3.89, and 3.57 molecules of the fluorophore per molecule of each antibody.

Immunoreactivity Measurement. Figure 1C shows the RPMI 1788 cell binding of ^{125}I-NuB2 in the presence of unlabeled NuB2, nonradioactive SIB-conjugated NuB2, SIB-$NuB2_I$, and SIB-$NuB2_{III}$. Unlabeled NuB2 almost completely inhibited the binding of ^{125}I-NuB2 to RPMI 1788 cells, and no significant differences were observed in the inhibitory curves among the nonradioactive SIB-NuB2, SIB-$NuB2_I$, and SIB-$NuB2_{III}$. Similar results were observed with AF-labeled antibodies (data not shown).

Localization of the Antibodies in RPMI 1788 Cells. Figure 2A shows the localization of NuB2, $NuB2_I$, and $NuB2_{III}$ in living cells when determined by confocal microscopy. All AF-labeled antibodies (green) were present on the cell membrane. Under the conditions, LysoTracker (red) was observed in acidic compartment of the cells such as endosome and lysosome.

In Vitro Cell Binding and Retention. When the radiolabeled antibodies were incubated with the RPMI 1788 cell at 1 h at 37 °C, [^{125}I]SIB-$NuB2_I$ and [^{125}I]SIB-$NuB2_{III}$ showed 1.4 and 4.3 times higher cell-associated radioactivity than [^{125}I]SIB-NuB2 (Figure 2B). When the radiolabeled antibodies were incubated in the presence of 1000-fold excess of NuB2, significant reduction in the cell-associated radioactivity was observed with [^{125}I]SIB-NuB2 and [^{125}I]SIB-$NuB2_I$. However, [^{125}I]SIB-$NuB2_{III}$ still registered high cell-associated radioactivity. Similarly, higher cell-associated radioactivity was observed

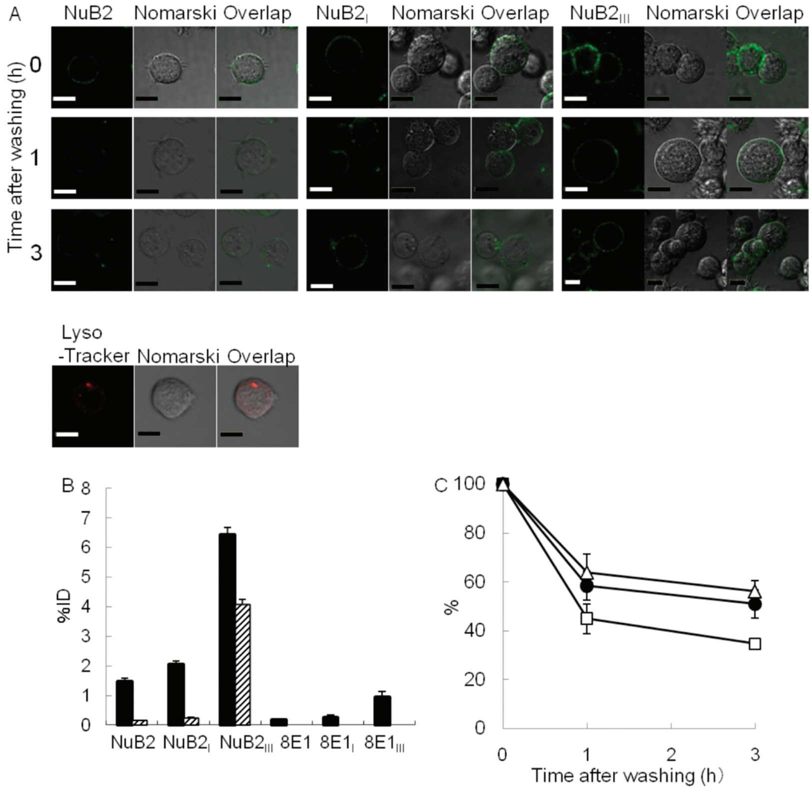


Figure 2. Cellular processing of [^{125}I]SIB-NuB2s. (A) Confocal microscopy to detect NuB2s localization (green) in RPMI 1788 cells as a function of incubation intervals at 37 °C; bars, 10 μm. (B) RPMI 1788 cell-associated radioactivity after 1 h incubation of [^{125}I]SIB-NuB2s at 37 °C (solid column). Similar experiments were performed in the presence of 1000-fold excess of NuB2 (hatched column). (C) Retention of [^{125}I]SIB-labeled NuB2s ([^{125}I]SIB-NuB2 (square), [^{125}I]SIB-NuB2$_I$ (solid circle), [^{125}I]SIB-NuB2$_{III}$ (triangle)) on RPMI 1788 cells after washing and reincubation in a fresh medium for 1 and 3 h (average of triplicates and SD).

in [^{125}I]SIB-8E1$_{III}$ when compared with [^{125}I]SIB-8E1 and [^{125}I]SIB-8E1$_I$.

Figure 2C shows the retention of [^{125}I]SIB-NuB2, [^{125}I]SIB-Nub2$_I$, and [^{125}I]SIB-NuB2$_{III}$ from RPMI 1788 cells as a function of reincubation times. While [^{125}I]SIB-NuB2 showed 44.3% and 34.2% of the initial radioactivity levels at 1 and 3 h postincubation, [^{125}I]SIB-NuB2$_I$ exhibited the cell-bound radioactivity of 59.1% and 51.2% at the same postincubation times. [^{125}I]SIB-NuB2$_{III}$ showed radioactivity levels of 67.8% and 61.3% at 1 and 3 h postincubation, respectively.

In Vivo Studies. The biodistribution of radioactivity after intravenous injection of [^{125}I]SIB-NuB2, [^{125}I]SIB-NuB2$_I$, and [^{125}I]SIB-NuB2$_{III}$ to normal mice is summarized in Table 1. Both [^{125}I]SIB-NuB2 and [^{125}I]SIB-NuB2$_I$ showed similar radioactivity levels in the blood, liver, kidney, and intestine. However, [^{125}I]SIB-NuB2$_{III}$ exhibited faster clearance of the radioactivity from the blood with higher radioactivity levels in the liver throughout the postinjection time examined. As a result, [^{125}I]SIB-NuB2$_{III}$ exhibited higher liver-to-blood ratios of the radioactivity.

Table 2 shows the biodistribution of the radioactivity after simultaneous injection of [^{125}I]SIB-NuB2 and [^{131}I]SIB-NuB2$_I$ in SCID mice bearing RPMI 1788 cells. [^{131}I]SIB-NuB2$_I$ exhibited significantly higher radioactivity levels in the tumor than those observed with [^{125}I]SIB-NuB2 from 48 to 96 h postinjection. [^{131}I]SIB-NuB2$_I$ exhibited similar radioactivity levels in other tissues such as the blood, liver, kidney, and intestine when compared with [^{125}I]SIB-NuB2.

DISCUSSION

In this study, we estimate the ability of an octaarginine peptide, R_8, as an anchoring molecule to enhance target accumulation of radiolabeled antibodies. The spatial location of the anchoring molecules attached to an antibody was considered of importance to maintain antigen binding properties, specific binding, and biodistribution profiles of the parental

Table 1. Biodistribution of Radioactivity after Intravenous Injection of [^{125}I]SIB-NuB2, [^{125}I]SIB-NuB2$_I$, and [^{125}I]SIB-NuB2$_{III}$ in Normal Mice[a]

	time after injection		
	1 h	3 h	24 h
	[^{125}I]SIB-NuB2		
blood	26.51 (0.99)	22.37 (1.33)	12.63 (0.48)
liver	7.65 (0.49)	5.99 (0.34)	3.34 (0.29)
kidney	5.92 (0.43)	5.46 (1.68)	3.31 (0.15)
stomach[b]	0.56 (0.55)	0.84 (0.08)	1.27 (0.44)
intestine	1.30 (0.07)	1.69 (0.15)	1.36 (0.33)
liver/blood	0.29 (0.03)	0.27 (0.00)	0.26 (0.01)
	[^{125}I]SIB-NuB2$_I$		
blood	26.26 (0.83)	20.70 (0.14)	10.54** (0.61)
liver	7.03 (0.3)	5.72 (0.33)	2.81 (0.40)
kidney	6.85* (0.31)	5.38 (0.50)	3.37 (0.35)
stomach[b]	0.58 (0.06)	1.01* (0.07)	1.89 (0.95)
intestine	1.15 (0.17)	1.68 (0.04)	0.95 (0.16)
liver/blood	0.27 (0.01)	0.28 (0.02)	0.27 (0.03)
	[^{125}I]SIB-NuB2$_{III}$		
blood	20.32 (3.79)	15.45** (1.36)	8.08** (0.49)
liver	17.91** (0.42)	14.71** (0.46)	5.85** (0.69)
kidney	6.50 (0.73)	5.80 (0.57)	3.42 (0.17)
stomach[b]	0.86** (0.05)	1.30* (0.25)	1.66 (0.67)
intestine	1.01 (0.08)	1.42 (0.30)	0.82 (0.17)
liver/blood	0.90** (0.14)	0.96** (0.12)	0.72** (0.05)

[a] Tissue radioactivity is expressed as percent of injected dose per gram of wet tissue. Results are expressed as means (SD) of three animals each point. [b] Expressed as percent of injected dose per tissue. Significances determined by unpaired Student's *t*-test. * $p < 0.05$ compared to [^{125}I]SIB-NuB2. ** $p < 0.01$ compared to [^{125}I]SIB-NuB2.

Table 2. Biodistribution of Radioactivity after Intravenous Injection of [^{125}I]SIB-NuB2 and [^{131}I]SIB-NuB2$_I$ in RPMI1788 Cell-Bearing SCID Mice[a]

	time after injection			
	24 h	48 h	72 h	96 h
	[^{125}I]SIB-NuB2			
blood	14.05 (1.02)	8.64 (1.18)	4.79 (1.22)	3.02 (0.39)
xenograft	4.54 (0.68)	5.36 (1.03)	3.95 (1.08)	4.81 (0.78)
liver	5.01 (0.82)	3.04 (0.39)	1.68 (0.27)	1.01 (0.10)
kidney	4.56 (0.67)	3.09 (0.50)	1.70 (0.35)	1.08 (0.22)
stomach[b]	0.33 (0.05)	0.20 (0.04)	0.38 (0.06)	0.09 (0.01)
intestine	1.15 (0.13)	1.05 (0.18)	0.58 (0.11)	0.36 (0.08)
xenograft/blood	0.32 (0.06)	0.62 (0.09)	0.83 (0.03)	1.59 (0.35)
liver/blood	0.36 (0.07)	0.35 (0.01)	0.35 (0.04)	0.33 (0.04)
	[^{131}I]SIB-NuB2$_I$			
blood	14.60 (0.57)	8.17 (1.14)	4.58 (0.72)	2.78 (0.33)
xenograft	6.20* (0.92)	7.52* (1.19)	6.04* (1.21)	7.20** (1.32)
liver	4.33 (0.49)	2.42* (0.29)	1.39 (0.10)	0.80** (0.08)
kidney	4.78 (0.71)	2.95 (0.47)	1.57 (0.18)	1.03 (0.22)
stomach[b]	0.36 (0.06)	0.20 (0.04)	0.37 (0.07)	0.08 (0.01)
intestine	1.34 (0.19)	1.03 (0.18)	0.59 (0.08)	0.35 (0.09)
xenograft/blood	0.42 (0.07)	0.92** (0.11)	1.31** (0.11)	2.59* (0.64)
liver/blood	0.30 (0.03)	0.30** (0.01)	0.30 (0.03)	0.29 (0.03)

[a] Tissue radioactivity is expressed as percent of injected dose per gram of wet tissue. Results are expressed as means (SD) of five to six animals for each point. [b] Expressed as percent injected dose. Significances determined by unpaired Student's *t*-test. * $p < 0.05$ compared to [^{125}I]SIB-NuB2. ** $p < 0.01$ compared to [^{125}I]SIB-NuB2.

antibodies. It was speculated that the attachment of anchoring molecules close to an antibody molecule may cause less interaction with cell surface molecules or cell membrane of target cells, whereas attachment of linkers that place the anchoring molecules at a distance from the antibody molecule may increase nonspecific binding. We selected a butyryl-bis(aminocaproate) linkage between a maleimide moiety and an octaarginine peptide with an expectation that the octaarginine moiety would not interact directly with a cell but interact strongly after the specific binding of antibody takes place. Since the importance of the density of AR-CPP molecules in an antibody was well-documented (*10, 11*), two R_8-conjugated antibodies holding different numbers of R_8 per molecule of the antibody were prepared (Scheme 1).

The R_8 conjugation and subsequent iodination reactions did not affect the SE-HPLC profiles of NuB2 (Figure 1B). The three SIB-labeled NuB2s possessed similar immunoreactivity to the antigen-positive tumor cells (Figure 1C). However, the heavy conjugation of R_8 to NuB2 increased the bandwidth of isoelectric focusing and raised pI of the resulting NuB2$_{III}$ (Figure 1A). The reaction of R_8 derivatives and an antibody can be better understood by considering the example of a perfectly random reaction (*29*). Poisson distribution can be used to predict how the R_8 molecules will be distributed among the individual antibody molecules. According to the calculation (Figure 1D), the majority of NuB2$_I$ contained up to 2 molecules of R_8 per molecule of NuB2. On the other hand, NuB2$_{III}$ consisted of a variety of conjugates ranging from 1 to 7 R_8 molecules per molecule of NuB2 with over 65% of NuB2$_{III}$ containing more than 3 R_8 molecules per molecule of NuB2, which accounts for the wide bandwidth of the isoelectric focusing.

The effect of R_8 conjugation levels on the target cell binding was assessed using CD20 positive cells. [^{125}I]NuB2$_{III}$ exhibited high cell-bound radioactivity levels even in the presence of a 1000-fold excess of cold NuB2. Since the cell-bound radioactivity levels of [^{125}I]SIB-8E1$_{III}$ were much lower than those of [^{125}I]SIB-NuB2$_{III}$, the majority of the cell-associated radioactivity of [^{125}I]NuB2$_{III}$ would be attributable to the specific antigen binding, followed by the strong interaction of the R_8 moieties with the cell. The low cell-bound radioactivity of [^{125}I]SIB-NuB2$_I$ in the presence of excess NuB2 suggested slower interaction kinetics of R_8 moieties with the cell, following the specific binding. However, when the dissociation rates from the cells were compared, not only [^{125}I]SIB-NuB2$_{III}$ but also [^{125}I]SIB-NuB2$_I$ exhibited much longer residence times in the CD20 positive tumor cells than [^{125}I]SIB-NuB2 (Figure 2C). The confocal microscopy confirmed that both NuB2$_I$ and NuB2$_{III}$ were present on the cell membrane (Figure 2A). These studies supported the hypothesis that the conjugation of anchoring molecules that possess strong interaction with cell surface molecules or cell membrane prolonged the retention of radiolabeled antibodies on tumor cells by fixing the antigen-bound antibodies on target cells, although the binding kinetics of anchoring molecules with the cell was affected by their density. This also suggested that further improvement of spatial location of the R_8 molecule may increase the binding kinetics.

Despite favorable anchoring ability, the heavy conjugation of R_8 affected biodistribution profiles of the parental antibody. As shown in Table 1, [^{125}I]SIB-NuB2$_{III}$ displayed faster clearance from the blood and higher accumulation in the liver in normal mice, as also observed in prior studies (*30*). On the other hand, both [^{125}I]SIB-NuB2 and [^{125}I]SIB-NuB2$_I$ displayed similar biodistribution (Table 1). These results reinforced the importance of the number of R_8 molecules attached per molecule of an antibody to retain its in vivo behavior (*10, 11*) and showed that the present conjugation of up to 2 R_8 molecules per molecule of NuB2 did not induce any significant changes in biodistribution. Recently, we have observed that the biodistribution of PAMAM dendrimers is predominantly determined by their net molecular charge as measured by both zeta potential and pI values (*31*). The present study also suggests that the determination of net molecular charge of AR-CPP-modified antibodies may be useful to predict their pharmacokinetics.

The biodistribution studies in tumor-bearing SCID mice were investigated with [^{125}I]SIB-NuB2 and [^{131}I]SIB-NuB2$_I$ since they exhibited similar pharmacokinetics in normal mice. To minimize individual differences, the biodistribution studies were conducted after injection of a mixed solution of [^{131}I]SIB-NuB2$_I$ and [^{125}I]SIB-NuB2. As shown in Table 2, the role played by R_8 conjugation on tumor accumulation was clearly demonstrated in the biodistribution studies, where [^{131}I]SIB-NuB2$_I$ exhibited significantly higher radioactivity levels in the xenograft than those of simultaneously administrated [^{125}I]SIB-NuB2 with no significant increase being observed in the radioactivity levels of blood, liver, and other tissues between the two. When considering the in vitro studies, these results would be attributable to the delayed dissociation rates of [^{131}I]SIB-NuB2$_I$ from the target cells in vivo.

In conclusion, the findings in this study indicated that the conjugation of anchoring molecules to an antibody that possesses a strong interaction with cell surface molecules or cell membrane enhanced accumulation in target tissues by fixing antigen-bound antibodies on target cells. This study also showed that conjugation of up to 2 molecules of R_8 per molecule of NuB2 satisfied both target-specific binding of antibodies and strong cell binding property of R_8. Further optimization of the conjugation chemistry would be required to provide uniform conjugates with appropriate linkage structure between R_8 and an antibody. These findings strongly suggested the application of AR-CPP to enhance specific accumulation of antibodies in target for more effective RIT.

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