Localization of Radiolabeled Antimyeloid Antibodies in a Human Acute Leukemia Xenograft Tumor Model

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ABSTRACT

Acute myeloid leukemia is an attractive disease to treat with radiolabeled antibodies because it is radiosensitive and antibody has ready access to the narrow cavity. In order to evaluate potentially useful radiolabeled antibodies against human acute myeloid leukemia, we have developed a nude mouse xenograft model using the human acute leukemia cell line, HEL. Mice with s.c. xenografts of HEL cells received infusions of radiiodinated anti-CD33 antibody. Examination of the biodistribution of the antibody showed that uptake in the s.c. tumor was maximal [16.9% injected dose (ID)/g at 1 h after infusion] following infusion of 1—10 µg of antibody and decreased following infusion of 100 µg (6.5% ID/g) at 1 h presumably as a result of saturation of antigen sites. The radiolabel was poorly retained in tumor (4.5—8.2% ID/g at 24 h after infusion). These results were consistent with in vitro studies demonstrating rapid internalization and catabolism of the anti-CD33 antibody. Uptake in tumor could be improved by using either a radiolabel that is retained intracellularly, 131I-DTPA (18.5% ID/g at 24 h), or by targeting a surface antigen that does not internalize upon antibody binding, CD45 (20.5% ID/g after 24 h). These results indicate that this model system will be useful in evaluating the interaction of radiolabeled antibodies with human acute myeloid leukemia cells in an in vivo setting.

INTRODUCTION

Recent studies have demonstrated that radiolabeled monoclonal antibodies against antigens expressed on lymphohematopoietic malignancies can localize to areas of tumor and have antitumor effects in both experimental systems and early clinical trials (1—10). Leukemia is a particularly attractive disease to treat using radiolabeled antibody because the disease is radiosensitive and there is ready access of antibody to the marrow space. However, because the antibody has such ready access to the target tissues, the amount of antibody required to saturate cell surface antigenic sites may be much different than that required to target lymphomas or other solid tumors. In addition, the retention of antibody may also be influenced by characteristics of the antigen, including the rapid internalization and metabolism of antibodies seen following antigen binding.

In clinical studies, we have studied marrow uptake and retention of an antibody against the CD33 antigen the expression of which is virtually restricted to the hematopoietic system (10). We observed that a relatively small amount (0.05 mg/kg) of a 131I-labeled anti-CD33 antibody is rapidly taken up by marrow but is not retained over time. To further the evaluation of radiolabeled antibodies against human leukemia, we have developed a xenograft model using human acute myeloid leukemia cells. This model uses s.c. inoculation of tumor cells in standard BALB/c nude mice with the development of a s.c. tumor mass. Using this model we have examined the tumor uptake and retention of radiolabeled antibody and have evaluated ways to increase localization to AML cells.

In the present report, the effect of antibody dose and labeling technique on uptake and retention of antibody in tumor were studied. In additional studies, we examined the uptake of an 131I-labeled antibody against the CD45 antigen which, unlike the CD33 antigen, does not modulate internally upon antibody binding.

MATERIALS AND METHODS

Animals. Male BALB/c nude mice (8—12 weeks old; 25—30 g) were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). All mice were housed in a specific pathogen free environment using disposable microisolator units at less than six mice per unit. Bedding, food, water, and cages were autoclaved prior to use. Cages were changed daily for the first week in each experiment and thereafter 2—3 times a week. Drinking water was acidified (2% HCl) and contained 0.30 mg/ml neomycin. All procedures were performed in a laminar air flow hood.

Leukemia. The HEL cell line (11) established at the Fred Hutchinson Cancer Research Center was maintained in culture in RPMI media containing 10% bovine calf serum. HEL cells (4 x 10^6) in 0.2 ml of media were injected s.c. in one flank of each mouse. Antibody was infused 8—12 days after injection when a palpable tumor nodule 0.5—1.5 cm in diameter was present. All experiments with in vitro cells were performed with exponentially growing cultures.

Monoclonal Antibodies. Monoclonal antibody p67 is a murine IgG1 antibody isolated in our laboratory which recognizes the human CD33 antigen (12). Monoclonal antibody BC8 is a murine IgG1 which recognizes the human CD45 antigen (13). The cell line producing BC8 was kindly provided by Dr. Claudio Anasetti. Two murine anti-idiotypic IgG1 antibodies, 31.A and DT, against Thy-1.1 and a human lymphoma idiotype, respectively, were used as nonspecific controls. Antibodies were trace labeled with 131I or 125I (ICN, Irvine, CA) using Iodo-Gen (Pierce Chemical Co., Rockford, IL) or 111In-DTPA (cyclic anhydride method) at a specific activity of 1.0 Ci/g or less as described previously (2, 14).

Immunoreactivity (percentage of counts able to bind at antigen excess) was determined in each experiment by binding to viable HEL tumor cells, as described previously (15). Briefly, immunoreactivity was determined by incubating serial dilutions (10^-6 to 10^-10 cells/ml) of target cells (HEL or control) with 4 ng/ml of radiolabeled antibody for 1 h at room temperature. Cells were centrifuged and the supernatant radioactivity was counted. Lineweaver-Burk analysis was used to calculate the immunoreactivity. Likewise, avidity was determined by incubating serial dilutions of radiolabeled antibody (1—0.01 µg/ml) with 10^5 target cells for 1 h at room temperature. Cells were centrifuged and washed once with buffer; the cell pellet was counted for radioactivity and

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Scatchard analysis was used to calculate the avidity (association constant) of the antibody and the number of binding sites per cell.

**Biodistribution Studies.** Most biodistribution experiments were performed with paired labels, either $^{111}$I plus $^{125}$I or $^{111}$In plus $^{125}$I. Animals received injections of a mixture of equivalent amounts of specific anti-myeloid antibody and control antibody via tail vein at 8–12 days after tumor inoculation (0.5–1.5-cm-diameter tumors). Animals were sacrificed at 1, 24, and 48 h after injection of antibody. Samples of blood, tumor, lung, liver, spleen, and kidney were weighed and counted in a gamma counter. The percentage of injected dose per g of tissue for each isotope was calculated by comparison to an aliquot of the injected material. Three to four mice were included in each group. For dose escalation studies a single labeling was performed and unlabeled antibody was added to achieve the desired total dose of antibody with a constant amount of radioactivity.

**Antigen Expression.** Expression of CD33 and CD45 by tumor cells was analyzed by indirect flow microfluorimetry. For *in vivo* studies, pieces of tumor nodules were minced and intact single cells were isolated on a Ficoll-Hypaque density gradient (specific gravity, 1.090). Single cell suspensions of *in vivo* or *in vitro* tumor cells were incubated with (a) no antibody; (b) FITC labeled GAM antibody; (c) control antibody plus FITC labeled GAM antibody; (d) p67 antibody plus FITC labeled GAM antibody; (e) BC8 antibody plus FITC labeled GAM antibody. All manipulations were performed with chilled reagents ($4^\circ$C, incubations done on ice) to minimize *in vitro* modulation. Cells were analyzed using a fluorescence activated cell sorter and scanner (EPICS 750; Coulter Electronics, Inc., Hialeah, FL) using a logarithmic amplifier.

**In Vitro Measurement of Antibody Metabolism.** The rate at which $^{125}$I-labeled antibody was internalized and degraded was measured *in vitro* as described previously (16). Briefly, HEL cells were incubated with $^{125}$I-labeled antibody (5 µg antibody/10⁷ cells) in a volume of 0.1 ml for 45 min on ice. Cells were then washed and placed in culture at $37^\circ$C. At 0, 1, 4, 10, and 24 h aliquots of the incubation mixture were assayed for cell associated radioactivity and supernatant radioactivity. The percentage of supernatant radioactivity precipitable with trichloroacetic acid was determined. Data were corrected for nonspecific binding by incubation with 100-fold excess unlabeled antibody.

**Statistical Analysis.** Data were analyzed using the SAS statistical package (SAS Institute, Cary, NC). Comparison among multiple doses were performed by analysis of variance. Comparisons in paired labeling experiments were performed using a paired t test.

**RESULTS**

**Tumor Growth and Antigen Expression.** Following s.c. injection of 3–4 × 10⁷ HEL cells, palpable myeloid tumors developed in 95% of animals by day 5–7 and grew exponentially to 0.5–1.0 cm in diameter by day 10. The tumors expressed both CD33 and CD45 antigen (Fig. 1). Fluorescence intensity on *in vivo* tumor cells was somewhat greater than that observed with *in vitro* cells, possibly because of detection of antibody bound *in vivo* nonspecifically to Fc receptors. Fluorescence from cells stained with anti-CD45 antibody was greater than with anti-CD33 antibody using HEL cells from either *in vivo* or *in vitro* sources, suggesting higher expression of CD45 compared to CD33. Tumors maintained in the mice for up to 5 weeks postinjection continued to grow and express the CD33 and CD45 antigens. However, areas of necrosis were apparent grossly in some tumors by 3 weeks after injection.

**Antibody Binding and Metabolism in Vitro.** The binding characteristics of the radiolabeled antibodies are shown in Table 1. Both the anti-CD33 and the anti-CD45 antibodies had good immunoreactivity (>90%). The avidity of the anti-CD33 antibody was higher than the anti-CD45 (4 × 10⁰⁶ versus 1.5 × 10⁶ liters/mol). Corresponding to the lower peak fluorescence on flow cytometry, there were fewer molecules of the radiolabeled anti-CD33 antibody bound per cell at saturation than of the anti-CD45 antibody (6 × 10⁴ versus 4.3 × 10⁵).

The two antibodies also differed in the rate at which they were metabolized following binding to HEL cell surfaces. The fate of cell surface antibody was examined *in vitro* by incubating cells with saturating amounts of $^{125}$I-labeled antibody, followed by washing and incubation for 24 h in antibody free media. The fraction of activity remaining cell associated, and the TCA precipitable as well as TCA soluble activities in the supernatant were determined over time (Fig. 2). There was a rapid decrease in cell associated activity of the $^{125}$I-labeled anti-CD33 antibody, with only 38% of counts remaining after 4 h of incubation. The decrease in cell associated activity coincided with an increase in TCA soluble activity in the supernatant, suggesting that anti-CD33 antibody was rapidly internalized and metabolized. In contrast, the majority of activity from the $^{125}$I-labeled anti-CD45 antibody remained cell associated and there was little evidence of catabolism over 24 h.

**Biodistribution of Radiiodinated Anti-CD33 Antibody.** The concentration (% ID/g) of $^{125}$I from labeled anti-CD33 antibody in tumor bearing mice (8–10 days after inoculation, 0.5–1.5-cm-diameter tumor) was determined at 1 and 24 h after infusion of 1, 10, or 100 µg of antibody. At 1 h after infusion of 1 or 10
these ratios were higher for the anti-CD33 antibody than for control antibody, these differences were not significant ($P = 0.06-0.26$). They progressively decreased following infusion of 10 or 100 $\mu$g and at the 100 $\mu$g dose tumor:tissue ratios of the anti-CD33 antibody were similar to those of control. In contrast, at 24 h after infusion, tumor:tissue ratios of the anti-CD33 antibody were similar to the control antibody at all doses in all organs except spleen.

CD33 Antigen Expression in Vivo. The basis for the poor retention of the anti-CD33 antibody was examined in vivo by using flow microfluorimetry to determine bound cell surface antibody and expression of CD33 on tumor cells at 1, 24, and 48 h after infusion of 100 $\mu$g of labeled antibody (Fig. 4). At 1 h after infusion, there was a significant amount of cell surface antibody as indicated by the higher fluorescence with the FITC-anti-mouse reagent compared to the untreated control tumor. Fluorescence increased after incubation with excess exogenous anti-CD33 antibody, demonstrating that unbound CD33 was present. Furthermore, the total amount of fluorescence was comparable to that with tumors from untreated controls. At 24 h after infusion, cell surface sites were nearly completely saturated with antibody, as indicated by the lack of increased fluorescence with exogenous anti-CD33, and the total fluorescence was less than seen with untreated tumor, suggesting that modulation had occurred. By 48 h, antibody was no longer detected on the cell surface and there was only a small increase in fluorescence with added anti-CD33. These results demonstrated that a 100 $\mu$g dose of antibody was capable of saturating tumor cells as suggested by the decreased tumor uptake shown in Fig. 3. In addition, they suggested that the reason why activity was poorly retained in tumor was that after antibody bound to the cell surfaces there was rapid modulation, internalization,

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Tissue} & \textbf{Antibody} & \textbf{1 $\mu$g} & \textbf{10 $\mu$g} & \textbf{100 $\mu$g} \\
\hline
\textbf{Blood} & Anti-CD33 & 0.46 & 0.45 & 0.11 \\
 & Cont & 0.19 & 0.36 & 0.10 \\
\hline
\textbf{Kidney} & Anti-CD33 & 2.34 & 2.01 & 0.76 \\
 & Cont & 1.17 & 1.43 & 0.74 \\
\hline
\textbf{Liver} & Anti-CD33 & 1.95 & 1.68 & 0.83 \\
 & Cont & 0.83 & 1.05 & 0.73 \\
\hline
\textbf{Lung} & Anti-CD33 & 2.04 & 2.15 & 0.55 \\
 & Cont & 0.91 & 2.0 & 0.50 \\
\hline
\textbf{Spleen} & Anti-CD33 & 2.73 & 2.61 & 1.33 \\
 & Cont & 1.10 & 2.25 & 1.95 \\
\hline
\end{tabular}
\caption{Tumor:tissue ratios (mean ± SEM of $^{125}$I-labeled anti-CD33 and $^{125}$I-labeled control antibody for 3-4 animals/group)}
\end{table}

Fig. 2. In vitro metabolism of anti-CD33 and anti-CD45. $^{125}$I-labeled antibodies were incubated on ice with in vitro grown HEL cells for 1 h. Cells were washed, resuspended, and incubated at 37°C. The percentage of initial activity remaining cell associated and percentage of supernatant activity present as TCA soluble were determined. Note the total radioactivity does not sum to 100%; the remainder was TCA precipitable radioactivity in the supernatant (presumably dissociated intact antibody). • •, cell associated cpm; O——O, TCA soluble cpm.

Fig. 3. Uptake of $^{125}$I-labeled anti-CD33 antibody in tumor. Tumor bearing mice received infusions of a mixture of 1, 10, or 100 $\mu$g of $^{125}$I-labeled anti-CD33 antibody plus an equivalent amount of $^{125}$I-labeled control antibody. Mice were sacrificed at 1 or 24 h after infusion; tumors were excised, weighed, and counted. Percentage of injected dose per g (mean of 3 animals) is shown: , anti-CD33; , control.

\textbf{Table 2 Tumor:tissue ratios (mean ± SEM of $^{125}$I-labeled anti-CD33 and $^{125}$I-labeled control antibody for 3-4 animals/group)}

\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Tissue} & \textbf{Antibody} & \textbf{1 $\mu$g} & \textbf{10 $\mu$g} & \textbf{100 $\mu$g} \\
\hline
\textbf{Blood} & Anti-CD33 & 0.47 & 0.37 & 0.63 \\
 & Cont & 0.52 & 0.40 & 0.28 \\
\hline
\textbf{Kidney} & Anti-CD33 & 1.98 & 1.65 & 2.11 \\
 & Cont & 1.37 & 1.9 & 1.17 \\
\hline
\textbf{Liver} & Anti-CD33 & 1.51 & 1.72 & 2.11 \\
 & Cont & 1.13 & 2.0 & 1.80 \\
\hline
\textbf{Lung} & Anti-CD33 & 2.18 & 1.19 & 1.68 \\
 & Cont & 1.18 & 1.0 & 2.29 \\
\hline
\textbf{Spleen} & Anti-CD33 & 2.13 & 1.81 & 1.61 \\
 & Cont & 1.18 & 1.98 & 1.25 \\
\hline
\end{tabular}

\textit{a} Ratios different among antibody doses, $P < 0.05$.

\textit{b} Anti-CD33 different from control, $P < 0.05$.
'25I-labeled antibody is shown in Fig. 7. Tumor uptake following infusion of 5 μg of the '25I-labeled anti-CD33 was quantitatively higher and retained longer than '25I-labeled anti-CD33 (18.5% ID/g versus 4.5% ID/g at 24 h; \( P < 0.01 \)). Although tumor uptake was substantially increased by '25I-DTPA label-

and metabolism similar to that seen in vitro. Two approaches were evaluated in an attempt to overcome this rapid metabolism: an antibody (anti-CD45) that is not internalized; and '111In-labeling of the anti-CD33 antibody.

**Biodistribution of 125I-labeled Anti-CD45 Antibody.** Following infusion of 100 g of the anti-CD45 antibody, specific antibody concentrations exceeded those of control at 1 h, and there was increasing uptake in tumor over the first 24 h [from 9.4 ± 3.0 to 21.2 ± 1.9% ID/g (Fig. 5)]. Concentrations in tumor were well maintained at 48 h (19.3 ± 1.6% ID/g). In contrast, there were no significant differences between anti-CD45 and control antibody concentrations in normal organs (data not shown).

Examination of tumors for cell surface antibody and expression of CD45 showed that there was antibody bound to the cell surface by 1 h after infusion and near saturation at 24 and 48 h (Fig. 6). Levels of CD45 expression remained comparable to those in untreated controls suggesting that there was little modulation. Thus, infusion of an iodinated antibody against a nonmodulating antigen expressed in higher density resulted in better initial localization to tumor than infusion of iodinated anti-CD33 antibody, and radionuclide was retained at the tumor site.

**Biodistribution of 111In-labeled Anti-CD33 Antibody.** The biodistribution of 111In-labeled anti-CD33 antibody compared to
Fig. 7. Biodistribution of $^{111}$In-DTPA labeled anti-CD33. Concentration (% ID/g) of $^{111}$In and $^{131}$I-labeled anti-CD33 or control antibody in tissues 24 h after infusion antibody are shown. Groups of animals received a mixture of 5 μg of $^{111}$In-labeled anti-CD33 antibody plus 5 μg of $^{131}$I-labeled anti-CD33 antibody or 5 μg of anti-CD33 antibody labeled with one nuclide plus 5 μg of control antibody labeled with the other nuclide. Columns, mean of 4 animals. □, $^{111}$In-labeled anti-CD33; ■, $^{111}$In-labeled control; ○, $^{131}$I-labeled anti-CD33; □, $^{131}$I-labeled control.

ing, there were also increased concentrations of both the $^{111}$In-labeled anti-CD33 and $^{111}$In-labeled control antibody in liver and kidney compared to $^{131}$I-labeled antibody.

**DISCUSSION**

Preliminary studies in humans have demonstrated that it is possible to use radiolabeled antibodies against the CD33 antigen to deliver radiation to marrow for treating acute leukemia (9, 10). However, these early studies suggest that the optimal dose of antibody for targeting cells residing in the marrow space may be substantially lower than those we have found optimal for targeting solid tumors including lymphoma (2, 4). In addition, the retention of $^{131}$I-labeled anti-CD33 antibody in marrow has been relatively short (median $t_{1/2}$, 19 h). This short retention is presumably due to rapid internalization and deiodination, as suggested by in vitro studies.

In order to examine the interaction of radiolabeled antibodies with human AML cells in vivo, we have developed a model human acute myeloid leukemia growing as a s.c. tumor in nude mice. Using the HEL cell line, we were able to reproducibly grow tumor nodules that continued to express human hematopoietic antigens. Using this model, we first examined the biodistribution of the p67 antibody against the CD33 antigen. Expression of this antigen is relatively restricted to myeloid leukemia and immature myeloid cells and thus has the potential for delivering radiation selectively to the marrow space. Small doses (1–10 μg) of $^{131}$I-labeled anti-CD33 antibody specifically localized to tumor, while increasing the dose of antibody to 100 μg saturated antigenic sites in the tumor and decreased specific uptake. Although low doses of iodinated anti-CD33 antibody localized to tumor, the activity was not retained. These results are similar to the uptake of $^{131}$I-labeled anti-CD33 antibodies in human marrow (9, 10). Studies of the fate of antibody bound to HEL cell surfaces in vitro and in vivo demonstrated that rapid modulation and degradation of bound antibody was the cause of this poor retention. These results suggest that alternative labeling chemistries that result in prolonged intracellular retention (17) will be required for antibodies that rapidly modulate.

The studies using $^{111}$In-DTPA labeled anti-CD33 antibody reported here confirmed that this labeling technique also has the potential for increasing tumor uptake when using antibodies against internalized antigens, presumably because the radiounclide is retained intracellularly. Similar differences between $^{111}$In-labeled and $^{131}$I-labeled anti-CD5 antibodies have been observed in studies of T-cell leukemia/lymphoma (18). The $^{111}$In-labeled antibody used in our study was taken up in significant amounts by the liver and kidney, presumably because of the presence of 15% free $^{111}$In and instability of the DTPA chelate. It is possible that this normal organ uptake can be minimized by the use of improved chelates (19).

An alternative approach toward improving uptake and retention is to use an antibody against an antigen that does not modulate, such as anti-CD45. In contrast to the anti-CD33 antibody, the anti-CD45 antibody was stably retained with up to 20% ID/g present in tumor 24 h after infusion.

The results of our initial studies suggest that this nude mouse model will be useful in evaluating radiolabeled antibodies against human AML, particularly with regard to the metabolism of the antibody. Although the kinetics of antibody localization to the tumor mass in our model was similar to that of antibody localization to marrow in humans, the extent to which this model will be useful in examining the pharmacology and dosimetry of radioimmunotherapy of leukemia is uncertain. For this purpose, models targeting normal marrow or syngeneic leukemia/lymphoma may be more appropriate (1–3, 20, 21).

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**REFERENCES**


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