ABSTRACT

Monoclonal antibodies against the Thy 1.1 differentiation antigen are ineffective in the treatment of transplanted AKR T-cell lymphoma once a palpable tumor nodule is present, due to the inability of the host to eliminate antibody-coated tumor cells. To overcome this limitation, we have evaluated the use of 131I-labeled anti-Thy 1.1 antibodies for the therapy of established AKR/J SL2 lymphoma (Thy 1.1+) nodules growing in congenic AKR/Cu mice (Thy 1.2*). In these experiments, 131I-anti-Thy 1.1 antibody specifically localized to a s.c. tumor with a mean of 6.5% of the infused dose per g of tumor at 24 h after infusion. The proportion of infused anti-Thy 1.1 antibody localizing to tumor was constant following antibody doses of up to 400 µg/animal. Antibody iodinated with up to 2 atoms of iodine per antibody of molecule maintained binding activity and localization to tumor equivalent to antibody labeled with less iodine. The concentrations of 131I-anti-Thy 1.1 in tumor would result in delivery of a mean of 1600 cGy to tumor following infusion of 500 µCi of 131I-labeled anti-Thy 1.1 antibody. In comparison, 500 µCi 131I-irrelevant antibody would deliver a mean of 380 cGy to tumor. Treatment of animals with palpable tumor nodules with 500 µCi 131I-anti-Thy 1.1 led to regression of the tumor nodule in 44% of animals, significantly prolonged survival, and cured two of five of the animals treated prior to the development of metastatic disease. In contrast, unlabeled anti-Thy 1.1 led to tumor response in 6% of animals, and up to 1000 µCi 131I-irrelevant antibody had no effect on tumor growth. Therapy was limited by the emergence of variant tumor cells lacking the target antigen and by bone marrow toxicity following 131I-labeled antibody doses of >1000 µCi/animal. These studies demonstrate that 131I-labeled monoclonal antibodies can have a significant antitumor effect in a situation where unmodified antibody is ineffective.

INTRODUCTION

Monoclonal antibodies against a number of tumor-associated antigens have been shown to inhibit the outgrowth of tumor cells when given shortly after tumor implantation (1, 7-9, 21, 24, 40, 42, 43). However, antibodies have not been effective in eliminating large numbers of tumor cells in experimental systems, and results of clinical trials with unmodified antibody in advanced disease have been disappointing (34). We have extensively studied the therapy of murine AKR T-cell lymphomas with monoclonal antibodies against the Thy 1.1 differentiation antigen (1, 7-9). With optimal administration schedules, anti-Thy 1.1 antibody was unable to completely prevent outgrowth of more than 3 x 10^6 SL2 lymphoma cells. The failure to eliminate large numbers of cells at the s.c. inoculation site occurred despite the presence of antibody on tumor cell surfaces and was a result of a limitation in the ability of host effector mechanisms to eliminate antibody-coated tumor cells. These studies therefore suggested that the treatment of large numbers of tumor cells would require that antibody be conjugated with a cytotoxic agent that is directly toxic to tumor cells, thus overcoming the need for a host effector mechanism to eliminate antibody-coated tumor cells.

In the present study, we have evaluated the use of 131I-labeled anti-Thy 1.1 monoclonal antibodies to deliver radiotherapy to murine lymphoma. As part of these studies, the ability of anti-Thy 1.1 antibody to localize to a s.c. AKR/J SL2 Thy 1.1+ tumor mass growing in congenic AKR/Cu Thy 1.2* mice was examined. Factors that might influence the concentration of antibody in tumor, including tumor size and host anti-Thy 1.1 immune response were evaluated. The influences of the quantity of administered antibody and of the iodination ratio of the labeled antibody on localization to tumor were both determined to define a therapeutic regimen. Based on these studies, therapeutic trials of iodinated antibody in mice with established tumor were then performed.

MATERIALS AND METHODS

Animals and Leukemias. Male AKR/J (Thy 1.1*) mice (6 to 8 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME) or from the Fred Hutchinson Cancer Research Center Animal Health Resources. Male AKR/Cu (Thy 1.2*) mice (6 to 8 weeks old, 25 to 30 g) were obtained from Cumberland Laboratories (Clinton, TN).

The isolation of the AKR/J, SL2 and AKR/Cu, SL1 T-cell lymphomas has been described previously (30). Lymphoma cells were passaged in vitro and in vivo by i.p. administration. SL2 lymphoma cells bind approximately 10^6 molecules of 125I-labeled anti-Thy 1.1 antibody to the cell surface at saturation.3 Cell surface Thy 1.1 antigen on SL2 does not modulate in vivo in the presence of anti-Thy 1.1 antibody; however, stable variant cells lacking the Thy 1.1 antigen do arise (1). SL2 cells have a doubling time of 24 h both in vitro and in vivo. In experimental studies, SL2 lymphoma cells were administered as single-cell suspensions of in vivo-passaged cells given s.c. in the flank in a volume of 0.1 ml.

Monoclonal Antibodies. Hybridoma cells secreting monoclonal antibody 31E6.4, a homogeneous murine IgG2b that reacts specifically with the T-cell differentiation antigen Thy 1.1, were a gift of Drs. Peter Lu (Fred Hutchinson Cancer Research Center) and Robert Nowinski (Genetic Systems Corp., Seattle, WA). Monoclonal antibody 7.2, a murine monoclonal IgG2b reactive with human lymphocytes, was a gift of Dr. Paul Martin (Fred Hutchinson Cancer Research Center). Monoclonal antibody G3G6 is a murine IgG2a antibody reactive with a human platelet-associated antigen. Antibodies 7.2 and G3G6 were used as irrelevant controls.

1 Supported in part by NIH Grant CA33477.
2 Recipient of an American Cancer Society Junior Clinical Faculty Fellowship. To whom requests for reprints should be addressed, at Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.
3 Unpublished data.
control antibodies for this study. Hybridomas were inoculated i.p. in syngeneic mice for the production of ascites.

Antibodies were purified from ascites by adsorption and elution from a Staph Protein A/SePharose column (Sigma Chemical Co., St. Louis, MO) using a pH step gradient (17). Eluted antibody was concentrated and diafiltered into phosphate-buffered saline (40 mM NaCl/8 mM NaH₂PO₄/2.7 mM KCl/1.5 mM KH₂PO₄, pH 7.2) using a PM-10 ultrafiltration membrane (Amicon Corp., Danvers, MA). Concentrated antibody was aliquoted and stored at -70 °C until use. Antibody concentration was determined using the Bio-Rad protein assay standardized with bovine γ-globulin (Bio-Rad, Richmond, CA) (10).

Iodination and Characterization of Labeled Antibody. Iodination was performed in 20-ml glass scintillation vials coated with 100 µg or 1 mg of lodogen (Pierce Chemical Co., Rockford, IL) depending on the quantity of antibody to be labeled, but generally in a ratio of 100 µg or 1 mg of lodogen per 100 µg or 1 mg of antibody. Antibody was diluted in phosphate-buffered saline to a volume of 1 ml in the lodogen-coated vial, and radiiodine (Na¹³¹I or Na¹²¹I; ICN, Irvine, CA) or a mixture of radiodiode and stable NaI was added. ¹³¹I had a specific activity of 12 to 25 Ci/mg iodine and ¹²¹I had a specific activity of 17 Ci/mg iodine as reported by the supplier. The vial was incubated at room temperature with intermittent agitation for 10 min. Unbound iodine was removed by passage over a Sephadex PD-10, G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ).

Approximately 50 to 60% of starting radioactivity was recovered bound to protein. Labeled antibody was consistently >98% precipitable with trichloroacetic acid. Radioactivity and protein concentration of the purified labeled product were determined, and iodination ratios (mol iodine/mol antibody) of the labeled antibody were calculated assuming: (a) proportional labeling of ¹³¹I or ¹²¹I and stable sodium iodide; (b) a specific activity of 15 CI/mg iodine and 17 CI/mg iodine for ¹³¹I and ¹²¹I, respectively; and (c) an antibody molecular weight of 160,000.

Avidity was determined from Scatchard plots of the binding of labeled antibody to viable SL2 leukemic cells (38). Known quantities of antibody were diluted in tissue culture media (RPMI 1640 plus 2% BSA) and incubated with 2 × 10⁶ AKR/J SL2 or 2 × 10⁴ AKR/Cu SL1 leukemic cells in microtiter plates in a total volume of 0.2 ml for 1 h at 37 °C. The cells were washed 3 times, and bound radioactivity was counted. Under these conditions, a maximum of 40% of labeled antibody 31E6.4 bound to SL2 cells. Less than 0.1% of labeled 31E6.4 bound to SL1 cells. Labeled 7.2 or G3G6 did not bind to SL2 or SL1 (<0.1%).

Binding Inhibition and Cytotoxicity. Analysis of serum samples to determine the presence of activity that would inhibit antibody binding to SL2 leukemic cells was performed by incubating serum diluted in RPMI 1640 plus 2% BSA with 2 × 10⁶ SL2 leukemic cells plus 125I-labeled 31E6.4 (70% of maximal binding) for 1 h at 37 °C. Cells were washed 3 times, and bound activity was counted. Serum inhibitory activity was quantitated by comparison with the inhibition produced by known quantities of unlabeled 31E6.4 under the same conditions (23).

Serum complement-dependent cytotoxicity was determined by ⁶⁷⁷Cr release assay using ⁶⁷⁷Cr-labeled SL2 cells as targets with a 10% lysis end point as described previously (19).

Immunofluorescence Studies. Tissue samples (tumor nodules or spleen) were minced in RPMI 1640 plus 2% BSA and viable cells from tumor nodules and spleen were isolated on a Ficoll-Hypaque density gradient (specific gravity, 1.090). Single-cell suspensions were incubated with: (a) anti-Thy 1.1 antibody followed by FITC-labeled rabbit anti-mouse IgG (Bionetics Laboratory Products, Litton Bionetics, Inc., Kensington, MD) to determine Thy 1.1 expression; (b) FITC-anti-mouse IgG alone to determine the antibody binding to the cell surface; (c) FITC-anti-mouse IgM to determine background fluorescence. Cells were examined by flow microfluorometry using the fluorescence-activated cell sorter (8-8 D FACS Systems, Becton Dickinson & Co., Sunnyvale, CA) or with a fluorescence microscope. Using the fluorescence histograms from the fluorescence-activated cell sorter, the quantitation of cell surface antibody resulting from in vivo binding was determined by comparing the location of the fluorescence peak from incubation of cells with FITC-anti-Thy 1.1 alone, representing an in vivo binding, to the location of the fluorescence peak obtained with preincubation with excess anti-Thy 1.1 antibody, representing the maximum amount of antibody able to be bound (see Footnote 1 for details).

Antibody Localization. Biodistribution studies were performed by the double isotope labeling method of Pressman (33). AL2 lymphoma cells (3 × 10⁶) were implanted s.c. in the flank of AKR/Cu mice. When a 0.5- to 1-cm-diameter tumor nodule was present (7 to 8 days after inoculation), a mixture of ¹³¹I-labeled anti-Thy 1.1 antibody and ¹²⁵I-labeled control antibody was infused i.v. via tail vein. At various times following infusion of the labeled antibodies, a blood sample was obtained, and mice were sacrificed with ether anesthesia. Tissues were excised, weighed, and counted in a multiple-channel gamma counter (Auto-Gamma Model 5330 spectrometer; Packard Instruments, Downers Grove, IL) to determine ¹³¹I and ¹²⁵I activity. ¹²⁵I counts were adjusted for decay of either ¹³¹I or ¹²⁵I. All results were expressed as the percentage of the administered dose per g of tissue (%/g) to allow ready comparison of the concentration of administered dose when varying quantities of antibody or varying iodination ratios were administered. Absolute concentration in tissues for a given infusion can be obtained by multiplying this value by the administered dose in µg or µCi to obtain µg antibody/g tissue or µCi/g tissue, respectively (see Footnote 5).

Radiation Dosimetry. Approximate potential radiation doses to various tissues from infusion of ¹³¹I-labeled anti-Thy 1.1 and ¹²⁵I-labeled IgG2a control antibody were calculated from the biodistribution of labeled antibody assuming uniform distribution of isotope within individual organs. The area under the biodistribution curve was determined from the mean %/g obtained for each antibody at 6 h and 1, 2, 4, 6, and 8 days by the trapezoidal integration method. Values for ¹³¹I-control antibody were obtained from the data for ¹²⁵I-control antibody correcting for decay of ¹³¹I, the initial concentration of radiolabeled antibody in tumor was assumed to be 0%/g, while integral concentrations of antibody in all other tissues were assumed to be equivalent to the values at 6 h, the latter being an approximate measure of plasma volume for each tissue. Radiation doses were then calculated for a 1000 µCi initial antibody dose by multiplying the integrated µCi h/g by the g/Cy/µCi-h, which has been tabulated by the Medical Internal Radiation Dose committee (13). For ¹³¹I, the value is 0.4165 for the total of all β-particles, low-energy X-rays, and Auger electrons, all of which are totally absorbed in the source organ where the isotope is deposited. The major γ-ray for ¹³¹I is 0.364 MeV and deposits 0.6465 g/Cy/µCi/h but this energy is poorly absorbed with only 10% deposited in a sphere with a radius of 3 cm. Thus, for the small organs in a mouse, the γ-component to the absorbed dose has been neglected.

We elected to calculate cumulative radiation doses by directly integrating the area under the biodistribution curves rather than by the effective half-life method (27) since for several tissues, particularly tumor, uptake of labeled antibody did not fit an exponential model. Direct integration will result in slightly higher calculated radiation doses for portions of the clearance curves that are concave and slightly lower doses for curves that are convex compared to the effective half-life method. The differences in calculated dose between the 2 methods are small compared to the differences between animals that would result from the variation in antibody concentrations in tissues (see "Results").

RESULTS

Antibody Localization

We first measured the biodistribution of radiolabeled antibody...
following a single i.v. infusion. The localization of 131I-labeled anti-Thy 1.1 antibody 31E6.4 and an 125I-labeled irrelevant IgG2a control antibody to tumor and normal organs is shown in Chart 1. The concentration of 131I-anti-Thy 1.1 antibody in tumor rose over the first 24 h to a mean of 6.5% of the infused dose per g of tissue (%/g) (range, 2 to 15), was maintained at this level for approximately 24 h, and declined exponentially (t1/2, 104 h) thereafter with a mean of 3%/g remaining at 8 days. In contrast, a mean of 1.5%/g (range, 0.1 to 8.0) of the control antibody was present in tumor at 24 h and remained relatively constant over 8 days (t1/2, 220 h).

Clearance of both 131I-anti-Thy 1.1 antibody and 125I-control antibody from blood, lung, liver, and kidney demonstrated approximately exponential decline over time, and clearance rates were similar among these tissues for both 131I-anti-Thy 1.1 (t1/2, 33 to 40 h) and 125I-control antibody (t1/2, 72 to 85 h). Muscle, thymus, testis, and brain contained only low levels of either antibody (<0.5%/g) at all time points (not shown). The increase in activity in the stomach and salivary gland over the first 24 h (2%/g) was probably due to a small percentage of antibody that was deiodinated. The concentrations of 131I-labeled anti-Thy 1.1 antibody in spleen and bone marrow were slightly higher than those of the control antibody, possibly as a result of binding to metastatic tumor cells.

The slower clearance of the 125I-control antibody from blood and normal tissues was presumably a result of different rates of metabolic clearance (41) rather than uptake by tumor since activity in tumor accounted for less than 1% of the infused dose (a mean of 6.5%/g in a 0.1-g tumor). In a subsequent experiment (not shown), an irrelevant IgG2b control antibody (antibody 7.2) was cleared from the blood and normal tissues at the same rate as the anti-Thy 1.1 antibody. Concentrations of the anti-Thy 1.1 and the IgG2b control antibody in tumor were similar to those for the anti-Thy 1.1 and IgG2a control antibody in tumor, respectively, as shown in Chart 1.

Variation of Antibody Concentration in Tumor

As noted above, there was a wide variation in the concentrations of both anti-Thy 1.1 and control antibody in tumor. An analysis of individual tumors in the paired labeling studies demonstrated that, at 24 h after infusion, there was a positive correlation (r = 0.8, P < 0.001) between concentration of 131I-labeled anti-Thy 1.1 antibody and concentration of 125I-labeled control antibody (Chart 2). A similar correlation of specific to control antibody concentration was present at 48 h after infusion (data not shown). Of note, the ratio of the concentration of 131I-anti-Thy 1.1 to 125I-control antibody, i.e., the specific localization, decreased from 10/1 to 20/1 at low concentrations to 2/1 as the absolute concentration of antibody in tumor increased.

The strong positive association of anti-Thy 1.1 antibody concentration with control antibody concentration suggested that variations in the permeability of the tumor vascular bed were responsible for the majority of the tumor-to-tumor variation in antibody concentration. Tumor size is one potential determinant...
of tumor vascularity and/or necrosis, and increasing tumor size has been associated with an increased accumulation of both antibodies (2, 28) and other serum proteins (4) following i.v. infusion. Although an attempt was made to study s.c. tumors of uniform diameter, actual tumor size as indicated by the weight of the excised tumor varied between 17 and 121 mg. Tumor weight had a weak positive association with concentration of 131I-anti-Thy 1.1 antibody in tumor (linear coefficient, 0.02%/g 131I; r = 0.8, P < 0.001).

Anti-Thy 1.1 Immune Response. Since in these studies there was an antigenic difference between the tumor (Thy 1.1) and host (Thy 1.2), it was possible that tumor-bearing animals would develop an immune response against the Thy 1.1 antigen. Such an immune response might result in circulating anti-Thy 1.1 antibody which would compete with infused 131I-labeled antibody. In addition, an anti-Thy 1.1 response might also result in the selection of variant lymphoma cells lacking the Thy 1.1 antigen.

Serum obtained from animals 7 days after tumor inoculation uniformly inhibited in vitro binding of 131I-anti-Thy 1.1 antibody to SL2 leukemic cells equivalent to a mean of 328 ng/ml (range, 166 to 473; control, <45) of anti-Thy 1.1 antibody 31E6.4. In addition, all sera contained complement-dependent cytotoxic activity with a mean titer of 1/3000 (range, 1/892 to 1/6200; control, <1/200). Thus, the binding inhibition was a result of free anti-Thy 1.1 antibody rather than circulating antigen, since circulating Thy 1.1 antigen would be expected to produce binding inhibition in vitro but would not be cytotoxic. However, there was no correlation (r = 0.1; P = 0.8) between the level of endogenous antibody and the localization of labeled anti-Thy 1.1 antibody to tumor 24 h after infusion of 1 mg of radiolabeled antibody.

Thy 1.1 expression by viable cells from s.c. tumor nodules from 10 animals 7 days after tumor inoculation was examined by indirect immunofluorescence. The s.c. nodules were predominantly composed of cells that expressed Thy 1.1; however, a minor population of antigen-negative cells comprising <15% of viable cells was present in 6 of 10 animals. As a result of the endogenous anti-Thy 1.1 antibody response, there were low levels of surface antibody (mean saturation, 2.9% of available sites; range, 0.5 to 5%) on cells that expressed Thy 1.1.

In contrast, 50 to 100% of metastatic lymphoma cells in the spleen 14 days after tumor inoculation failed to express Thy 1.1, accounting for the low concentrations of 131I-anti-Thy 1.1 in spleen noted in the biodistribution studies. Of note, the amount of cell surface antibody on those cells that expressed Thy 1.1 (mean saturation, 7.5%; range, 5.2 to 8.8%) was higher than that on cells from the s.c. nodule.

Thus, the antigenic differences between tumor and host resulted in an anti-Thy 1.1 immune response. However, endogenous antibody was present in low levels, and only small numbers of antigen-negative tumor cells were present in the s.c. nodule, suggesting that these 2 factors had little, if any, influence on the localization of infused antibody to the s.c. tumor.

Effect of Antibody Dose and Iodination Ratio on Localization to Tumor

The dose of radioisotope administered as labeled antibody depends on both antibody dose and iodination ratio, i.e., the average number of iodine atoms per antibody molecule. Therefore, the effect on antibody concentration in tumor of each of these variables was evaluated independently.

Effect of Antibody Dose. The biodistribution of quantities of anti-Thy 1.1 and control antibody labeled at low iodination ratio (II/Ab < 0.05) varying over a 250-fold range was examined 24 h after infusion (Chart 3). There was no significant change (P = 0.44) in 131I-anti-Thy 1.1 concentration in tumor as the 131I-anti-Thy 1.1 dose varied from 10 to 400 /¿g/animal. There was a significant decrease (P = 0.05) in 131I concentration in tumor as the 131I-anti-Thy 1.1 dose was increased from 400 to 2500 /¿g/animal. In contrast, there was no evidence (P = 0.18) of antibody dose influencing the concentration of 125I-labeled control antibody present in tumor over the range of 10 to 2500 /¿g/animal. There were no differences among doses on the proportion of antibody localizing to normal organs for either anti-Thy 1.1 or control antibody (not shown). The biodistribution over time of total antibody doses varying from 10 to 1000 /¿g/animal was similar to that shown in Chart 1. Thus, total 131I-anti-Thy 1.1 antibody doses of up to 400 /¿g/animal could be given with maximum localization to tumor. Infusion of 1000 /¿g/animal led to only a slight decrease in mean tumor antibody concentration compared to the maximum tumor concentration obtained following infusion of 1 mg of radiolabeled antibody.
Linear regression analyses were performed on tumor antibody concentration versus log antibody dose for: (a) 131I-Thy administered 7 to 8 days following tumor inoculation. Mean of the percentage of administered dose per g of tumor 24 h after infusion is shown for 131I-anti-Thy 1.1 of 400 μg (see Chart 3).

Effect of Iodination Ratio. The effect of increasing the iodination ratio (I/Ab) on antibody avidity, serum clearance, and localization to tumor 24 h after infusion is shown in Table 1. Avidity, clearance, and localization did not change with average I/Ab much less than 1 atom/molecule. Under this condition, the preparation is a mixture of unlabeled antibody and molecules with exactly 1 atom/molecule; there are no multiply labeled molecules. Labeling at an average I/Ab of 2 resulted in a shift in avidity and increased serum clearance from blood but had little effect on localization to tumor. Labeling at I/Ab > 2 led to a progressive decrease in avidity and increased serum clearance (P < 0.001). Localization to tumor progressively decreased from 5.66 ± 0.94%/g at I/Ab 0.01 to 1.97 ± 0.24%/g at I/Ab 15 (P < 0.01). The antibody concentration in tumor relative to blood (tumor/blood) remained constant over the range of I/Ab studied, suggesting that the decreased concentration in tumor was a result of the decreased blood levels of labeled antibody. The more rapid clearance from blood with increasing I/Ab appeared to result from uptake by the liver since liver/blood ratios progressively rose from 0.21 ± 0.03 at the lowest I/Ab to 0.5 ± 0.12 at I/Ab 15 (P = 0.03). Anti-Thy 1.1 antibody concentrations in other normal organs decreased with increasing iodination ratio, corresponding to the decrease in concentration in blood, while tissue/blood ratios for the normal organs remained constant (not shown).

A separate experiment (not shown) examining the effect of iodination ratio on biodistribution over time confirmed the results obtained at 24 h after infusion. In this experiment, antibody was labeled at I/Ab 0.05, 0.9, and 2.0. Labeling at I/Ab 2.0 resulted in slightly faster clearance (P < 0.05) from blood (t½ 36 h) than either lower I/Ab (t½ 46.5 h). There were no significant differences among the 3 preparations on antibody concentration or clearance rates from tumor or normal tissues other than blood.

Therapy with Radiolabeled Antibody

The studies described above provided a basis for therapeutic trials by demonstrating that the biodistribution of labeled antibody did not vary following infusion of up to 1000 μg of 131I-anti-Thy 1.1. Similarly, labeling at iodination ratios of up to 2.0 (equivalent to 24 μCi/μg) did not influence biodistribution. In the studies described below, we first determined the maximum tolerated dose of 131I-labeled antibody and then calculated the dose of radiation that this amount of 131I-anti-Thy 1.1 would deliver to tumor. Subsequently, the therapeutic effects of 131I-antibody infusion were examined.

Toxicity and Dosimetry. To determine the maximum nonlethal dose of 131I-anti-Thy 1.1 antibody, normal AKR/Cu mice received infusions of 500 to 2000 μCi of 131I-labeled anti-Thy 1.1 antibody. All mice survived following infusion of 500 or 1000 μCi of 131I-anti-Thy 1.1 antibody. Approximately 50% of animals died 15 to 19 days after infusion of 1500 μCi, with the remainder surviving >90 days, while infusion of 2000 μCi was lethal to 100% of animals, with death occurring 14 to 15 days after infusion. Histological examination of animals receiving 1000 μCi of 131I-labeled antibody showed severe hypoplasia of the bone marrow (approximately 15% normal cellularity) 14 days after infusion with recovery of marrow cellularity by Day 21. Gastrointestinal mucosa appeared normal. Thus, bone marrow toxicity appeared to be dose limiting.

An approximation of the relative radiation doses that would be delivered to tumor and critical normal tissues by infusion of 1000...
μCi of 131I-labeled antibody was calculated from the biodistribution curves shown in Chart 1 assuming uniform distribution of isotope in the tumor and 100% absorption of all emitted β-particles (Table 2). The assumption of total absorption of β-particles was based on the fact that the maximum range of the most abundant β-particle from 131I is 2 mm and the average range is only 0.45 mm, dimensions which are small compared to those of any tissue being examined, including tumor fragments. The s.c. tumor would receive a mean of approximately 3300 cGy from infusion of 131I-anti-Thy 1.1. Radiation doses to normal tissues ranged from 1350 cGy to lung to 250 cGy to bone marrow. In comparison, 1000 μCi 131I-labeled IgG2a control antibody would deliver 750 cGy to tumor.

Dosimetry calculations were performed by extrapolation of biodistribution data obtained at low specific activities based on the fact that the biodistribution of labeled antibody was largely unaffected by antibody dose or iodination ratio within very wide limits (antibody dose, 10 to 1000 μg; I/Ab, 0.02 to 2.0). However, 2 factors make the application of these calculations to the therapeutic setting uncertain: (a) the concentration of antibody varied greatly from animal to animal in tumor (see Chart 2) and to a lesser extent in other organs (see Chart 1); (b) in the localization experiments where tracer amounts of radioidine were infused, the s.c. as well as the metastatic tumor continued to grow exponentially. Following therapeutic amounts of radioidine, tumor growth slowed or halted in those animals with 131I-anti-Thy 1.1. Radiation doses to normal tissues ranged from 1350 cGy to lung to 290 cGy to bone marrow. In comparison, 1000 μCi 131I-labeled IgG2a control antibody would deliver 750 cGy to tumor.

Therapy of Established Tumor. The therapeutic effect of 131I-antibody was evaluated in animals with an established s.c. nodule 0.5 to 1.0 cm in diameter 7 to 8 days following tumor implantation (Table 3). Infusion of 500 μCi of 131I-anti-Thy 1.1 antibody led to complete disappearance of the s.c. nodule in 3 of 27 (11%) animals and partial regressions in an additional 9 of 27 (33%) animals. Infusion of 1000 μCi of 131I-anti-Thy 1.1 resulted in complete regression in 9 of 34 (26%) animals and partial regression in an additional 15 of 34 (44%) animals. In contrast, infusion of equivalent amounts of unlabeled anti-Thy 1.1 led to complete or partial regression of the nodule in only 2 of 34 (6%) animals, and infusion of up to 1000 μCi of 131I-labeled control antibody did not result in tumor regression in any mice. Increasing the dose of infused antibody to lethal amounts (1500 to 3000 μCi) led to tumor regression in both 131I-labeled anti-Thy 1.1 and 131I-labeled control antibody. See Table 3 for attained significance values.

The survival of animals treated with 500 to 1500 μCi radiolabeled antibody on Day 7 following tumor inoculation from a representative experiment is shown in Chart 4. Infusion of 500 μCi of 131I-labeled anti-Thy 1.1 led to a significant prolongation in survival compared to treatment with 131I-labeled control antibody, unlabeled anti-Thy 1.1 or unlabeled control antibody (P < 0.01). There was no difference in survival among the unlabeled anti-Thy 1.1, 131I-labeled control antibody, and unlabeled control antibody groups. Infusion of 1000 μCi 131I-labeled anti-Thy 1.1 did not prolong survival compared to controls while 1500 μCi is slightly shortened survival (0.05 < P < 0.1). Animals receiving 3000 μCi of either 131I-labeled antibody uniformly died within 12 days of anti-Thy 1.1 administration with marked wasting and diarrhea. Death from the gastrointestinal syndrome occurs between 3 and 4 days after mice are exposed to 1000 cGy whole-body radiation (32). The whole-body radiation can be estimated by the blood/weight ratio and was just under 1000 cGy for the 3000-μCi dose.

To determine the cause of death in animals receiving ≤1000 μCi of 131I-antibody, autopsy examinations were performed on the

\begin{table}
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\begin{tabular}{llll}
\hline
\textbf{Antibody dose} & \textbf{No. treated} & \textbf{No. with partial regression} & \textbf{No. with complete regression} & \textbf{No. with complete and partial regression} \\
\hline
None & 19 & 0 & 0 & 0 \\
Anti-Thy 1.1 & & & & \\
1000 μCi & 6 & 0 & 0 & 0 \\
1500 μCi & 6 & 0 & 0 & 0 \\
3000 μCi & 6 & 0 & 0 & 0 \\
\hline
\end{tabular}
\caption{\textbf{131I-Antibody therapy: established tumor}}
\end{table}

Pooled results of 4 studies of treatment 7 to 8 days after inoculation of 2 x 10³ SCL2 leukemic cells are shown (mean tumor diameter, 0.8 cm). Partial regression was defined as reduction in both tumor diameters or 1 diameter and height on at least 2 subsequent days. Total antibody doses, 100 to 1200 μg; specific activity, 2 to 8 μCi/μg.
tumor-bearing animals 14 days after treatment with radiolabeled antibody. Animals receiving ≤500 μCi of 131I-labeled anti-Thy 1.1 antibody had extensive metastatic lymphoma. The bone marrow contained varying amounts of lymphoma, but hematopoiesis was otherwise normal. Animals treated with 1000 μCi 131I-anti-Thy 1.1 antibody also showed extensive metastatic lymphoma; in addition, the bone marrow in the majority of animals (6 of 8) was completely aplastic. Gastrointestinal mucosa appeared normal in all animals receiving ≤1000 μCi of labeled antibody. Thus, death in animals treated with 500 μCi of labeled antibody was a result of progressive metastatic lymphoma. In animals treated with 1000 μCi, death was a result of a combination of tumor growth and radiation toxicity to the bone marrow.

To evaluate the possibility that progressive tumor growth in spite of therapy was due to the presence of cells lacking the target antigen, expression of Thy 1.1 by viable cells in s.c. tumor nodules and metastatic tumor in the spleen was examined following therapy with 131I-labeled antibody (Table 4). All animals with less than complete regression following infusion of 500 or 1000 μCi of 131I-labeled anti-Thy 1.1 had persistence of cells expressing Thy 1.1 in the s.c. nodule. In contrast, metastatic spleen cells from all evaluable animals (9 of 9) treated with 500 to 1000 μCi 131I-anti-Thy 1.1 failed to express Thy 1.1. Such antigen-negative metastases were also present in control animals; however, 8 of 16 of these animals also had metastatic cells that continued to express Thy 1.1 (P = 0.01).

Since the cause of death in animals that had complete regression of the s.c. nodule following treatment with 500 μCi 131I-anti-Thy 1.1 antibody was the emergence of antigen-negative metastatic cells, we subsequently attempted therapy with labeled antibody 2 days after tumor inoculation, potentially prior to the metastatic spread of antigen-negative cells (Chart 5). All animals had a small (3- to 4-mm-diameter) s.c. nodule at the time of antibody treatment. Infusion of 500 μCi 131I-anti-Thy 1.1 again resulted in significant (P = 0.02) prolongation of survival compared to untreated controls and cure in 2 of 5 animals. Unlabeled anti-Thy 1.1 or 500 μCi 131I-irrelevant antibody again had no effect on tumor growth or survival.

**DISCUSSION**

Radioisotopes are attractive agents for antibody conjugate therapy for a number of reasons. Methods for labeling antibodies with isotopes are well established, and radioisotopes have been demonstrated to be able to produce antitumor effects in thyroid cancer. In addition, since cell damage is produced by an emitted particle rather than the isotope itself, internalization of labeled antibody is not required, and radiolabeled antibody bound to a cell will deliver radiation to adjacent cells which lack the target antigen.

In the present studies, we have used 131I-labeled antibody to treat established murine lymphoma, a situation where unconjugated antibody has little effect (1, 9). While a number of investigators have reported specific localization of radiolabeled antibody to tumors, the majority of these studies have been designed to study diagnostic imaging of tumors and have reported ratios of activity in tumor to activity in normal tissues at time points optimal for external imaging (3, 5, 6, 11, 12, 14, 15, 18, 20, 25, 29, 36, 37, 39). However, the requirements for therapy with radiolabeled antibodies are different than those for imaging, and only a limited number of studies of radiolabeled antibody for therapy have been reported (16, 19, 26-28, 31, 35). These differences in requirements exist because the absolute concentration of radioisotope in tumor over time is critical for therapy, and a relatively low ratio of tumor to normal tissue concentration (e.g., 1.5 to 2.0) may be sufficient for successful therapy of a radiosensitive tumor. In contrast, the relative concentration of isotope in tumor versus other tissues is more important for purposes of imaging, and high tumor/tissue ratios are desirable.
even if the absolute concentrations of isotope-antibody conjugate are low.

In the present studies, 131I-labeled anti-Thy 1.1 antibody specifically localized to a s.c. tumor mass in concentrations sufficient to deliver a mean of approximately 3300 cGy to the tumor following infusion of 1000 μCi 131I-anti-Thy 1.1. These results are comparable to the radiation doses of 450 cGy observed by Rostock et al. (35) following infusion of 500 μCi of 131I-labeled anti-ferritin antibody in a rat hepatoma model (35). There was also specific concentration of 131I-anti-Thy 1.1 in spleen and bone marrow (see Chart 1).

Although there was specific concentration of anti-Thy 1.1 antibody in tumor, both the specific anti-Thy 1.1 and control antibody concentrations in tumor were quite variable and highly correlated. A minor portion of the tumor-to-tumor variation was related to differences in tumor size with increasing tumor size associated with higher concentrations of both specific and control antibodies, an observation that has been made previously (12, 14, 16, 28, 37), and results from an increase in nonspecific accumulation of serum proteins (2, 4). Although low levels of endogenous anti-Thy 1.1 antibody were present, such endogenous antibody did not account for the tumor-to-tumor variation in antibody concentration since the variability was present following even high doses (1000 μg) of antibody. Similarly, the small numbers of antigen-negative cells present in the s.c. tumor did not account for the major differences in tumor antibody concentration. Thus, unidentified factors, presumably related to biological differences in tumor vascularity and/or necrosis, were responsible for the majority of the tumor-to-tumor variation noted in this and other studies (3, 12, 15, 18).

Antibody dose had only a minor influence on the concentration of radiolabeled anti-Thy 1.1 antibody in tumor with infusion of doses from 10 to 400 μg/animal achieving equivalent proportions of the infused dose in tumor. The low levels of endogenous anti-Thy 1.1 antibody presumably did not influence antibody concentration in tumor since even a 10-μg dose would achieve serum levels at 24 h (1600 ng/ml) that were in excess of the endogenous antibody (328 ng/ml). The concentration of 131I-anti-Thy 1.1 antibody in tumor following 2500 μg animal decreased compared to lower doses, probably as a result of saturation of surface Thy 1.1 sites in the s.c. tumor. In previous studies, 3200 μg/animal were found to be completely saturate all Thy 1.1 sites in a s.c. tumor even when the target antigen was widely distributed on normal T-cells (1).

Iodination ratios of an average of >2 iodine atoms/molecule, equivalent to 24 μCi/μg, led to decreased concentrations of 131I-anti-Thy 1.1 antibody in tumor compared to lower iodination ratios. The decrease in tumor concentration appeared to be a consequence of an increase in the rate of clearance from the serum since tumor/blood ratios were unaffected by iodination ratio. Presumably, highly labeled molecules were removed from the serum by the liver, and the remaining less highly labeled molecules bound to tumor in a relatively normal manner. The parallel decrease in avidity and antibody concentration in tumor suggests that changes in avidity may be a useful predictor of the maximum iodination ratio possible to retain maximum localization to tumor. Presumably, this will differ for different antibodies. Other investigators have also observed more rapid clearance of highly labeled antibody compared to the same preparation labeled with less iodine (28, 37).

Therapy studies demonstrated a clear, dose-related antitumor effect of 131I-anti-Thy 1.1. Infusion of 500 μCi of 131I-labeled anti-Thy 1.1 in tumor-bearing animals resulted in complete disappearance of nodule in 11% of animals and a total response rate of 44%. Survival was prolonged when antibody was given 7 days after tumor inoculation and cures were achieved when antibody was administered 2 days after tumor inoculation. In contrast, up to 1000 μCi of 131I-labeled irrelevant antibody had no effect on tumor growth in spite of slower clearance of the control antibody from the blood, while unlabeled anti-Thy 1.1 led to a tumor response in only 6% of animals.

Importantly, 131I-labeled anti-Thy 1.1 antibody was able to eliminate small metastatic deposits of cells that expressed Thy 1.1. All animals treated with 500 or 1000 μCi 131I-anti-Thy 1.1 had metastatic cells in the spleen that uniformly failed to express Thy 1.1. The prolongation of survival in animals treated with 500 μCi 131I-anti-Thy 1.17 days after tumor implantation probably resulted, in part, from the elimination of the metastatic cells that expressed Thy 1.1. However, at most 50% of metastatic cells expressed Thy 1.1. Elimination of only these cells would be expected to result in a 1- to 2-day prolongation in survival, given the tumor-doubling time in vivo of 24 h. Since an 11-day prolongation in median survival was observed, it is likely that a reduction in the number of antigen-negative metastatic tumor cells also occurred. Presumably, significant radiation was delivered to antigen-negative cells adjacent to those expressing Thy 1.1. Although the presence of antigen-negative metastases in the present study was a consequence of the host anti-Thy 1.1 response, the antigenic heterogeneity of human tumors is well established. The ability to deliver therapy to variant cells adjacent to those expressing the target antigen is an important theoretical advantage of radiolabeled antibodies compared to other potential antibody conjugates, e.g., toxins.

Infusion of 1000 μCi of 131I-labeled anti-Thy 1.1 did not prolong survival in spite of an increase in complete response rate of the s.c. tumor due to bone marrow toxicity. Bone marrow toxicity in tumor-bearing animals appeared greater than in normal animals, probably as a result of 131I-antibody binding to metastatic lymphoma in the marrow as indicated by the specific uptake of antibody 1.1 in the marrow shown in Chart 1. Hematological toxicity from radiolabeled antibody has been identified as dose limiting in early clinical trials of tumors that do not metastasize to marrow (26, 27, 31), and bone marrow toxicity is probably unavoidable when treating tumors that do metastasize to the marrow. Such toxicity can potentially be overcome by bone marrow transplantation, and bone marrow support would allow dose escalation to the point of gastrointestinal toxicity. Whether such therapy with radiolabeled antibodies will have an advantage over the doses of external radiation given in current regimens requiring bone marrow transplantation remains to be determined.

Although we were able to demonstrate a significant antitumor effect, complete regression of the s.c. nodule occurred in only 26% of animals treated with 1000 μCi 131I-anti-Thy 1.1. Failure to eliminate the s.c. nodule was a result of insufficient 131I-anti-Thy 1.1 antibody in tumor since surviving tumor cells continued to express Thy 1.1. Thus, achievement of improved results requires an increase in antibody concentration in tumor relative to normal organs. In these initial studies, we have used a single dose of whole antibody. However, the prolonged retention of antibody in tumor compared to the concentration in blood and
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