Therapy of Small Subcutaneous B-Lymphoma Xenografts with Antibodies Conjugated to Radionuclides Emitting Low-Energy Electrons

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ABSTRACT

Purpose: Radionuclides emitting low-energy electrons (Auger and conversion electrons of <50 keV) are potentially useful for cancer therapy when conjugated to an antibody, because they can irradiate the cell to which they bind while producing relatively little irradiation of surrounding cells and tissues. We showed previously the ability of such antibody conjugates to treat micrometastatic, disseminated human B-lymphoma in a severe combined immunodeficient mouse model using an anti-CD74 antibody. In this study, we have evaluated the ability of such conjugates to treat s.c. tumors.

Experimental Design: Severe combined immunodeficient mice were injected s.c. with Raji, Daudi, or RL B-lymphoma human tumor cells. Antibodies to CD74, CD20, or HLA-DR were radiolabeled with 111In or 125I and injected i.v. at various times starting at day 5, and tumor growth was monitored. Controls included the testing of unlabeled antibodies, labeled nonreactive antibodies, and a combination of the two.

Results: Therapy of s.c. B-lymphoma was more difficult than therapy of tumor cells that had been injected i.v. Although large, macroscopic tumors were not effectively treated, therapy was effective on s.c. Daudi tumors on day 5, and tumor growth was monitored. Controls included the testing of unlabeled antibodies, labeled nonreactive antibodies, and a combination of the two.

Conclusion: These results extend previous evidence that antibody conjugates with emitters of low-energy electrons can be effective therapeutic agents for micrometastatic cancer.

INTRODUCTION

There is increased interest in the use of radiolabeled antibodies for cancer therapy due in part to the recent approval of 90Y and 131I anti-CD20 antibodies for therapy of B-cell lymphoma. However, there remains uncertainty about the optimal radionuclide for this purpose. Moreover, the choice of the optimal radionuclide is likely to depend on the details of the particular situation, such as the tumor burden and the tumor type. Although most of the focus has been on β-particle emitters, these are most appropriate for killing substantial tumor masses. It is well known that as the tumor size becomes smaller, β-particles become increasingly less efficacious due to their long path lengths, because only a small fraction of the radiation is absorbed by the targeted tumor (1–3). Although relatively low-energy β-particles can be selected to treat small tumors, even 177Lu, which emits the lowest-energy β-particle of the nuclides widely used (average energy 149 keV, range in tissue 288 μm) has an optimal tumor target diameter for curability of 2.0 mm (2). To efficiently treat micrometastases or single tumor cells, it is necessary to use radiation having a shorter path length. To kill single cells, which can be considered the limiting case, the optimal electron energy for radionuclides deposited on the cell surface or in the cytoplasm is ~20 keV (range in tissue 8.9 μm; refs. 3–5). Electrons with approximately this energy include Auger electrons and some of the internal conversion electrons, which are emitted by radionuclides decaying by electron capture or internal conversion and which we collectively refer to as low-energy electrons (LEE). Some conversion electrons have relatively high energies (>100 keV), and these will not have the localized energy deposition that is desired. LEE emitters have less nonspecific toxicity than β-particle emitters both in vitro and in vivo by a factor of ~5 to 10 (6, 7). The disadvantage of LEE emitters is that they are weak, so a large number of decays are required to kill a cell. However, we have shown that sufficient decays can be delivered to tumor cells if antibodies to high-density antigens are used, and the specific activity of the conjugates is high (6, 8–10). α-Particles also have a relatively short path length and therefore are suitable for therapy of micrometastases. Moreover, they have a much higher level of energy deposition than LEE emitters (11). However, there are problems with the use of α-particle emitters due to limited availability and to the short half-life of most of the available radionuclides. The path length of α-particles (50 to 100 μm/L) is considerably greater than that of the optimal LEEs and much larger than a cell diameter, so they would be expected to have somewhat greater nonspecific toxicity. Although progress in the use of α-particle emitters has been made (12, 13) and there are many ongoing studies, evaluation of LEE emitters is also warranted.

Antibody conjugates of LEE emitters were shown to efficiently and specifically kill single cells in vitro (6, 8, 9). In vivo, they were effective therapeutic agents in a severe combined immunodeficient (SCID) mouse xenograft model of lymphoma.
disseminated B-cell lymphoma (Raji cell line), using an anti-CD74 antibody (7), under conditions in which 90Y conjugates had a much weaker effect. However, in the tumor model used, the antibody was injected i.v. 5 days after i.v. tumor inoculation, so the tumor burden was low, and the tumor cells would be expected to be relatively accessible to antibody. To extend these results, we have now investigated therapy of small s.c. tumors. Initial experiments again used a 5-day interval between tumor inoculation and antibody injection: 5-day-old s.c. tumors were in fact slightly less susceptible to therapy than 5-day-old i.v. tumors. However, effective therapy of s.c. Daudi tumors with 111In anti-CD74 antibody was obtained. [The Raji cell line, which was used in the previous study as a disseminated tumor (7), was less sensitive than Daudi cells to this form of therapy when grown s.c.] Moreover, doses lower than the maximum tolerated dose (MTD), and therapy as late as day 24 after tumor inoculation, were still effective. If two doses of antibody were given, a statistically significant therapeutic effect was obtained even at day 36 after inoculation of Daudi cells. Experiments were also done with other antibodies (anti-CD20 and anti-HLA-DR). This is important because the processing pathway of anti-CD74 antibodies is unique (14); therefore, results obtained with this antibody may not be representative of results that would be obtained with more typical antibodies binding to the cell surface. Anti-CD20 antibodies are clinically relevant, because, as noted, therapy with radiolabeled anti-CD20 antibodies is now an established therapeutic approach. With an anti-CD20 antibody and the B-lymphoma cell line RL injected s.c. into SCID mice, strong and specific therapy was obtained with 125I and 111In labels. The β-particle emitter 111In also had a specific therapeutic effect in this model, but it seemed to be less potent than the 111In conjugate. Although most experiments used a 5-day interval between injection of the tumor and the antibody, therapy was still effective with a 16-day interval. Both of the tumors used in these experiments grew slowly, so they were still small at the time of therapy (Daudi tumors at day 36 were thin discs <2 mm in diameter). These data show that small metastases are susceptible to this form of therapy.

MATERIALS AND METHODS

Cell Lines. The cell lines used were the B-cell lymphomas Raji, RL, and Daudi. The origin of these lines and the culture conditions were described previously (14, 15). The RL cell line is available from the American Type Culture Collection (Rockville, MD), although we obtained it elsewhere. This was derived from a patient with diffuse, large cell non-Hodgkin’s lymphoma, is negative for EB virus, and therefore is more typical of B-lymphomas in the United States than the Burkitt lymphomas Raji and Daudi. Cell lines were tested routinely for Mycoplasma contamination using the Mycotect assay (Life Technologies, Grand Island, NY) and were negative.

Antibodies and Radiolabeling. Anti-CD74 antibody (LL1, an IgG1) was supplied by the Antibody Production Facility at Immunomedics, Inc. (Morris Plains, NJ). The source of L243 (anti-HLA-DR, an IgG2a) was described previously (14, 16). The hybridoma producing 1F5 (anti-CD20, an IgG2a) was recloned from a stock obtained from the American Type Culture Collection as described (9). Nonreactive control antibodies were the IgG1 MN-14 (also provided by Immunomedics) and the IgG2a MX352a. Antibody purification was on an affinity column of Sepharose linked to protein A (Amersham Pharmacia, Piscataway, NJ). Antibodies were labeled with 125I and 111In using the chelator ITC-benzyl-diethylenetriaminepentacetic acid as described previously in detail (8). Labeled preparations were analyzed by instant thin-layer chromatography or by gel filtration high-performance liquid chromatography or both by methods that have been described (17) to determine the level of radioactivity not bound to the antibody, which was always <10% and usually <5%. Representative preparations of radiolabeled antibodies with each radiolabel were tested for immunoreactivity (% bindable) by incubating with a large excess of cells. Control tubes had excess unlabeled antibody added to block specific binding and to therefore indicate the level of nonspecific binding: specific binding was calculated by subtraction. The range of specific binding was as follows: LL1, 53.0% to 71.7%; 1F5, 50.6% to 74.8%; and L243, 56.3% to 58.6%. Such variation in immunoreactivity could have some effect on the data presented, but this would be at most a small effect and it would not significantly alter any of the conclusions.

Mice and Immunotherapy. Female or male SCID/NCr mice were obtained from the Animal Production Program of the National Cancer Institute (Frederick, MD). Their care was in accord with institutional guidelines. We note that the SCID mouse model is demanding, because these mice are twice to thrice more sensitive to radiation than normal mice (18). At 4 to 6 weeks of age, mice were shaved on the back and injected with 2.5 × 106 lymphoma cells s.c. The mice were monitored weekly for tumor growth. The tumor size was measured in three dimensions, and volume was estimated as the product of the three dimensions. Mice were sacrificed when tumors reached a size of 3 cm³, but most of the graphs show the time required for tumors to reach a size of 1 cm³. This size was selected arbitrarily, but the results shown do not depend on the particular size selected, because nearly all tumors grew at a relatively constant rate once they appeared for both of the tumor models used. The typical growth rate of Raji, Daudi, and RL tumors in SCID mice was described previously (19). For therapy, groups of 8 to 10 mice were injected at various times with radiolabeled antibodies i.v. into the tail vein. For experiments with IgG2a antibodies, mice were coinjected with ~0.2 mg nonreactive IgG2a to block uptake by Fc receptors, which otherwise causes variably rapid blood clearance (20). The IgG2a used in most of the studies was ascites of the hybridoma PK136, which had an IgG2a concentration of 2.08 mg/mL; 0.1 mL was injected per mouse. This hybridoma was obtained from American Type Culture Collection (HB-191) and was grown as ascites by Taconic Laboratories (Germantown, MD). This antibody reacts with a mouse alloantigen, CD161c, and was raised in a (C3H × BALB/c)F1 mouse, so it will not react with cells of the SCID mice, which have a BALB/c background. In some early experiments, the source of normal mouse IgG2a was 0.1 mL normal CF1 mouse serum (Charles River Laboratories, Wilmington, MA) containing 1.86 mg IgG2a/mL. IgG2a concentrations were determined by an ELISA assay as described (20). Experiments were terminated after 6 months, and all remaining mice at this time were normal and
apparently tumor free. Statistical comparisons between treatment groups used the log-rank test.

RESULTS

Determination of the MTD. The MTD is defined as the dose at which all of the treated mice survive with a weight loss of <20% and was determined in groups of five SCID mice without tumors. In preliminary experiments, mice were injected with serial 2-fold dilutions of the antibody. Subsequently, when the range was identified, doses were reduced incrementally by 20%. The MTD was determined separately for each of the antibody subclasses used (i.e., IgG1 and IgG2a). For IgG2a, irrelevant IgG2a (~200 µg) was coinjected to prevent blood clearance via the high-affinity Fcγ receptor as noted above. We showed previously that inclusion of the irrelevant IgG2a caused slower blood clearance and an increase in the MTD (20). The values of the MTD are given below under the individual experiments.

Therapy of S.c. B-Lymphoma Xenografts with Radiolabeled LL1. Attempted therapy of s.c. Raji xenografts in SCID mice with $^{111}$In-LL1 was based on therapy results with the disseminated tumor model (7) as well as on strong in vitro cytotoxicity obtained with this antibody-cell line combination (6, 8). In these previous studies, cpm bound at various time points was determined, cumulative decays per cell were calculated, and the rad dose delivered to the nucleus from the cell surface and/or cytoplasm was estimated using subcellular S values: this was consistent with the level of toxicity observed (6). Therapy results, when the antibody was given on day 5 after s.c. tumor inoculation, are shown in Fig. 1A. There was only marginally significant protection from tumor growth ($P$ are in the figure legend), which is much less dramatic than the protection obtained with a disseminated tumor (i.v. injection; ref. 7). Still, tumor growth was totally prevented in 3 of 10 mice. In an attempt to increase the therapeutic effect, the antibody injection was given on day 3 rather than day 5. In this case, a much stronger protective effect was obtained, which was statistically significant ($P < 0.005$; Fig. 1B). In these experiments, >90% of the mice that did not survive had large tumors of >1.0 cm$, but occasional mice were found dead with s.c. tumors <1 cm$ presumably due to tumor growth at other sites. Because this occurred in some of the control mice (2 of 20 mice), it was not a result of the radioactivity injected. Regardless of the cause of death, which is unknown, the frequency of such deaths was low enough such that the conclusions of the experiments are not affected.

Therapy with Daudi tumors was then attempted, because Daudi cells are more sensitive than Raji cells to cytotoxicity with radiolabeled antibodies (9) probably because they express a wild-type $p^{53}$ gene (21). Therapy of s.c. Daudi xenografts, with antibody injected i.v. on day 5, is shown in Fig. 2A. Treatment with 250 µCi $^{111}$In-LL1 totally prevented tumor growth, and all treated mice remained tumor free for the duration of the experiment. In contrast, all control mice had large tumors by 12 weeks. Treatment with a control nonreactive antibody, labeled similarly, had no significant therapeutic effect, and all of these mice also developed large s.c. tumors. Of the three LL1-treated mice that did not survive, all were found dead of unknown causes, with no sign of tumor growth. Because two of them died at week 20 or later, this does not seem to be due to radiation toxicity, unless a very delayed effect. Although we do not know if these mice died from tumor growth or from other causes, these late deaths do not affect the conclusions of the study. In addition, it should be noted that such deaths were not observed in most other similar experiments (such as the experiments described in Fig. 2B and C).

Subsequent experiments with s.c. Daudi tumors tested doses of $^{111}$In-LL1 lower than the MTD. Figure 2B shows results with serial dilutions of the antibody, again given at day 5 after tumor inoculation. A dose of 100 µCi (data not shown) or 33 µCi, which is only 13% of the MTD, totally prevented tumor growth. A dose of 11 µCi had a weak effect that was not statistically significant. Note that, in Fig. 2B, Daudi tumors grew in only 7 of 10 control mice. Although occasional mice did not develop tumors in this series of experiments, it was unusual for 3 of the 10 mice in the control group to remain tumor free. Still, this did not prevent observation of a strong therapeutic effect, highly significant statistically. Also included in this figure is a test of unlabeled LL1, at a dose of 10 µg, which is higher than the protein dose injected in any of the $^{111}$In therapy experiments: this had no effect. [However, a humanized IgG1 version of LL1, used at much higher doses, was therapeutically effective in disseminated xenograft models of B-lymphoma and other B-cell malignancies (22)].

In another experiment, therapy was started at later times after tumor inoculation. Figure 2C shows that therapy as late as

![Fig. 1](https://example.com/fig1.png)
Day 24 totally prevented tumor growth, although therapy at day 36 had no effect. Three to four control mice were dissected on the days on which antibodies were injected to determine the typical tumor size at the time of therapy. As noted above, this tumor grows slowly; in this particular experiment, which was typical, some tumors first reached a size of 1.0 cm\(^3\) at week 8 and 90\% of the tumors reached this size by week 12. Consistent with this slow growth, no tumors were visible macroscopically (after dissection) at day 17. At day 24, tiny s.c. tumor nodules were seen, which were 1 to 2 mm in diameter but very flat and attached to the mesentery. Even at day 36, there were no tumors visible without dissection. On dissection, three of four mice had multiple tiny tumor masses clustered on the mesentery, <2 mm in diameter, and very thin. These were too small to be excised and weighed. One conclusion from these dissections is that this tumor develops as multiple, closely spaced tumors that fuse together when they become larger.

In an attempt to treat slightly larger tumors, an experiment was done using two doses of \(^{111}\)In-LL1. The first injection of 250 \(\mu\)Ci (the MTD) was given on day 36, a time at which a single dose had no therapeutic effect in the previous experiment. A second dose was given 14 days later (on day 50); this time point was chosen arbitrarily based primarily on the 3-day half-life of the \(^{111}\)In and the hypothesis that the second injection should not be delayed too much or the tumors would be too large for effective therapy. Because the MTD that could be given on day 50 was unknown, varying doses were given to different groups of mice (200, 160, 128, or 102 \(\mu\)Ci). Results of these experiments are shown in Fig. 3. At the time of the first antibody injections, no tumors were visible. However, three tumors were evident 1 week later, and four other tumors were evident within 2 weeks. Thus, the first antibody injection was shortly before tumors became visible. We note that the tumors in this experiment grew slightly faster than in the experiment shown in Fig. 2C (by ~1 week). The single dose of \(^{111}\)In-LL1 had a slight protective effect in this experiment, which was marginally significant statistically (\(P < 0.1\)). By including a second injection, significant protection was obtained (\(P < 0.01\)) and 30\% of the mice were permanently tumor free. The second dose did not produce substantial toxicity as indicated by the body weight of the mice, which was measured weekly. Except for one mouse that had a 20.0\% loss of body weight (at 11 weeks), the maximum body weight loss was 14.3\%, and all of the body weight losses were recovered in 2 to 3 weeks. All four of the doses tested had essentially the same effect; therefore, Fig. 3 shows results with only the highest and lowest doses. In a
statistical comparison of mice given one or two doses, the significance was marginal for the individual groups ($P < 0.1$). However, when all of the mice given two doses were combined, the results with this group were significantly different from the group given one injection only ($P < 0.05$).

**Therapy with Radiolabeled Anti-CD20 and Anti-HLA-DR Antibodies.** To establish the generality of these results, it was necessary to test antibodies to other antigens. This is particularly true because of the unusual processing pathway of anti-CD74 antibodies as described previously (7). Accordingly, therapy experiments were done with a radiolabeled anti-CD20 antibody (1F5) and an anti-HLA-DR antibody (L243). Both of these antibodies, radiolabeled, are able to kill target cells efficiently in vitro (9). L243 is comparable in potency to LL1, whereas 1F5 is somewhat less potent due to a lower level of antibody uptake. One difference between these two antibodies and LL1 is that they are IgG2a antibodies, whereas LL1 is an IgG1. This is a significant difference for two reasons: First, rapid blood clearance of IgG2a antibodies usually occurs in SCID or nude mice, a consequence of the low level of endogenous IgG2a and IgG2b in these animals (20, 23, 24). This rapid clearance from the blood seems to be due to uptake by the high-affinity Fcγ receptor, CD64. To circumvent this problem, the mice were coinjected with an irrelevant IgG2a antibody at a dose of ~200 μg per mouse. As described previously (20), this use of normal IgG2a significantly affects the MTD: the MTD is higher when IgG2a is coinjected despite the longer circulation of the radiolabeled antibody in the blood probably because some of the antibody cleared from the blood is taken up in the bone marrow, which increases toxicity. The second problem with IgG2a antibodies is that they frequently have antitumor activity as unconjugated antibodies due to the fact that they are active in antibody-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity (25). This activity is most potent for high-density antigens, and both CD20 and HLA-DR are present at a high density on the target cells used, especially HLA-DR. Although this factor, of course, increases the therapeutic effect of the antibodies, it makes it more difficult to show a specific therapeutic effect of a radiolabeled conjugate. However, the antibody protein doses used in these experiments were not very high (5 to 40 μg per mouse), which reduces the effect of the unconjugated antibody. In any case, it is necessary to test the unconjugated antibody as a control, and this was included in most of the experiments described below. 1F5 had little or no protective effect, but only 10 μg L243, given on day 5 after tumor inoculation, totally prevented growth of s.c. Daudi tumors. Therefore, it was not possible to test radiolabeled L243 in this particular xenograft model.

Therapy in the systemic (i.v.) Raji model was evaluated first as done previously with LL1 (7). Therapy was attempted with 1F5 and L243 conjugated to $^{111}$In, $^{125}$I, or $^{131}$I. This was based on previous in vitro cytotoxicity experiments, in which these antibodies strongly killed Raji cells (9). The level of killing in these experiments was consistent with the radiation dose delivered as calculated from the cumulative decays per cell and the cytoplasmic $S$ values (10). Although the therapy data suggested some weak protective effects, effective and statistically significant therapy was not obtained (data not shown). In one experiment, $^{111}$In-1F5 was significantly more effective than unlabeled antibody ($P < 0.025$, log-rank test), but 60% of the mice treated with $^{111}$In-labeled 1F5 were paralyzed by day 39 and another 20% were paralyzed by day 67, meaning that the protection obtained was minor. 1F5 was then tested for therapy of the RL cell line growing s.c. in SCID mice. This model was selected because, in the killing of four B-cell lymphomas with $^{125}$I-1F5, RL cells displayed the highest specificity index, due to strong killing with 1F5, and weak killing with the nonreactive antibody (26). Because RL cells did not produce consistent paralysis when injected i.v. into SCID mice (or any other indication of tumor growth), in the systemic tumor model, we could test only a s.c. tumor model, also in SCID mice. Tumors in this system develop slowly; tumors first appeared in most of the mice at weeks 3 to 5 but grew slowly until ~6 to 7 weeks when the growth rate accelerated. After rapid growth began, a size of 1 cm$^3$ was reached in 2 to 3 weeks. Radiolabeled antibody was injected on day 5 after tumor inoculation. Results with $^{111}$In, $^{125}$I, and $^{131}$I conjugates are shown in Fig. 4. In the experiments with RL cells, no mice died without a large tumor of $>1.0$ cm$^3$. With $^{111}$In, at the MTD, all 10 treated mice remained tumor free for the duration of the experiment (22 weeks; Fig. 4A). The control groups showed that the effect was largely specific and dependent on the radiolabel. Unlabeled 1F5, at the same dose, was tested in a previous experiment and had little or no protective effect (data not shown). $^{111}$In-labeled nonreactive antibody had a small effect, with tumor growth delayed ~5 weeks, but this difference was not statistically significant. Because both unlabeled 1F5 and radiolabeled nonreactive antibody can potentially have a effect, separately, and because a combination of the two is likely to have enhanced activity, it is necessary to also test a mixture of the two to prove the specificity of radiolabeled 1F5 therapy. This control is shown in Fig. 4A using 8.8 μg 1F5: the mixture was slightly more effective than the labeled nonreactive antibody alone. Although the difference between these two groups was not statistically significant, the group with unlabeled 1F5 plus $^{111}$In nonreactive antibody was significantly protected relative to the untreated control group ($P < 0.05$). However, the group treated with $^{111}$In-1F5 was protected much better than any of the control groups ($P < 0.005$).

With $^{125}$I conjugates (Fig. 4B), only 3 of the 10 mice treated with $^{125}$I-1F5 at the MTD developed tumors. These three tumors developed early, but the other seven mice all remained tumor free for the duration of the study, which was 26 weeks. Nine of 10 control mice developed large tumors (>1.0 cm$^3$) by week 12, and the last mouse of this group did so at 16 weeks. The difference between groups was highly significant ($P < 0.005$). Other groups were treated with control reagents, including the same amount of unlabeled 1F5 (11.5 μg per mouse), $^{125}$I-labeled irrelevant IgG2a antibody, or a combination of both. All of these control groups were not significantly different from the untreated control group, although small effects were observed, as shown. The group treated with $^{125}$I-1F5 was significantly different from any of the control groups ($P < 0.05$).

Similar experiments were done with the $\beta$-particle emitter $^{131}$I. As shown in Fig. 4C, $^{131}$I-1F5 at the MTD also had a specific therapeutic effect ($P < 0.001$, compared with the uninjected group). In this experiment, there was some therapeutic effect from the $^{131}$I-labeled nonreactive antibody ($P < 0.05$, compared with the uninjected group) but markedly less than the
therapy obtained with $^{131}$I-1F5. Unlabeled 1F5 alone (1.7 $\mu$g per mouse) had no significant therapeutic effect. Treatment with combined $^{131}$I-labeled nonreactive antibody plus unlabeled 1F5 was slightly more effective than either of these reagents used alone but still was much less effective than $^{131}$I-1F5 ($P < 0.005$).

We can conclude that therapy is largely antigen specific and is dependent on the radiolabel, although there is a significant nonspecific effect of the $^{131}$I radiation. Results of the experiments with different radiolabels were also compared statistically: $^{111}$In-1F5 was significantly more effective than $^{131}$I-1F5 ($P < 0.05$), whereas $^{111}$In-1F5 was only marginally more effective than $^{125}$I-1F5 ($P < 0.10$), and there was no significant difference between the two iodine radiolabels.

The therapeutic potential of $^{111}$In-1F5 was further evaluated by testing doses lower than the MTD and by injecting the antibody at later times after tumor inoculation. As shown in Fig. 5A, with a dose of one third of the MTD (93 $\mu$Ci), tumors developed in four of eight mice at the same rate as in control mice, but the other four mice remained tumor free for the duration of the study, which was 25 weeks. The difference from the control group was statistically significant ($P < 0.05$, log-rank analysis). The lower dose of 31 $\mu$Ci (one ninth of the MTD) also prevented tumor growth in four of eight mice, but the tumors that developed appeared slightly faster than in the 93 $\mu$Ci group, which caused the difference from the control group to be of borderline significance ($P < 0.1$). Figure 5B shows that the MTD injected as late as day 16 after tumor inoculation still totally prevented tumor growth ($P < 0.001$, relative to control). This study used a 20% reduction in the injected dose (to 226 $\mu$Ci) based on indications that the dose of 280 $\mu$Ci $^{111}$In antibody, which was determined previously to be the MTD, might cause borderline toxicity in some experiments. Although in this experiment we did not examine the size of day 16 tumors, tumor masses (visible without dissection) are never seen at this time, and it is very likely that tumors at this time would be microscopic. We have not yet determined the latest time at which therapy is effective.

**Fig. 4** Therapy of s.c. RL tumors in SCID mice with 1F5 (anti-CD20 antibody) conjugated to $^{111}$In (A), $^{125}$I (B), or $^{131}$I (C). Groups of mice were injected with RL tumor cells and treated with the radiolabeled antibody on day 5 (○). Control mice were untreated (●). Other control groups received a nonreactive antibody (MX352a) conjugated in the same way and injected at the same dose (△), unconjugated 1F5 at the same protein dose (▽), or a mixture of unlabeled 1F5 plus the radiolabeled nonreactive antibody at the same $\mu$Ci dose (△). Mice without large tumors, as indicated, had no detectable tumor for the duration of the experiment, which was 22 weeks in A and 26 weeks in B and C. The dose injected was 280 $\mu$Ci $^{111}$In, 235 $\mu$Ci $^{125}$I, and 55 $\mu$Ci $^{131}$I. $^{131}$I. Results with radiolabeled 1F5 are representative of two experiments done with each radiolabel.

**Fig. 5** Therapy of s.c. RL tumors in SCID mice with $^{111}$In-1F5. A, varying doses were injected at day 5 after tumor inoculation. Doses injected were 93 $\mu$Ci, one third of the MTD (●), or 31 $\mu$Ci, one ninth of the MTD (○). The control group contained 18 mice that received no antibody (●). Other control groups received a nonreactive antibody (MX352a) conjugated in the same way and injected at the same dose (△), unconjugated 1F5 at the same protein dose (▽), or a mixture of unlabeled 1F5 plus the radiolabeled nonreactive antibody at the same $\mu$Ci dose (△). Mice without large tumors, as indicated, had no detectable tumor for the duration of the experiment, which was 22 weeks in A and 26 weeks in B and C. The dose injected was 280 $\mu$Ci $^{111}$In, 235 $\mu$Ci $^{125}$I, and 55 $\mu$Ci $^{131}$I. $^{131}$I. Results with radiolabeled 1F5 are representative of two experiments done with each radiolabel.

therapy obtained with $^{131}$I-1F5. Unlabeled 1F5 alone (1.7 $\mu$g per mouse) had no significant therapeutic effect. Treatment with combined $^{131}$I-labeled nonreactive antibody plus unlabeled 1F5 was slightly more effective than either of these reagents used alone but still was much less effective than $^{131}$I-1F5 ($P < 0.005$).

We can conclude that therapy is largely antigen specific and is dependent on the radiolabel, although there is a significant nonspecific effect of the $^{131}$I radiation. Results of the experiments with different radiolabels were also compared statistically: $^{111}$In-1F5 was significantly more effective than $^{131}$I-1F5 ($P < 0.05$), whereas $^{111}$In-1F5 was only marginally more effective than $^{125}$I-1F5 ($P < 0.10$), and there was no significant difference between the two iodine radiolabels.

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Fig. 4 Therapy of s.c. RL tumors in SCID mice with 1F5 (anti-CD20 antibody) conjugated to $^{111}$In (A), $^{125}$I (B), or $^{131}$I (C). Groups of mice were injected with RL tumor cells and treated with the radiolabeled antibody on day 5 (○). Control mice were untreated (●). Other control groups received a nonreactive antibody (MX352a) conjugated in the same way and injected at the same dose (△), unconjugated 1F5 at the same protein dose (▽), or a mixture of unlabeled 1F5 plus the radiolabeled nonreactive antibody at the same $\mu$Ci dose (△). Mice without large tumors, as indicated, had no detectable tumor for the duration of the experiment, which was 22 weeks in A and 26 weeks in B and C. The dose injected was 280 $\mu$Ci $^{111}$In, 235 $\mu$Ci $^{125}$I, and 55 $\mu$Ci $^{131}$I. $^{131}$I. Results with radiolabeled 1F5 are representative of two experiments done with each radiolabel.
DISCUSSION

In this study, we have further established the utility of LEE emitters, conjugated to antibodies, as therapeutic agents for B-cell lymphoma. Because positive results were obtained with an anti-CD20 antibody as well as with an anti-CD74 antibody, we can conclude that the rapid, high-level internalization that occurs with anti-CD74 antibodies is not required in order for this approach to be effective. Although we have not yet been able to effectively treat large, macroscopic tumor masses, treatment of micrometastatic tumors is an important clinical application. Considering that effective therapy could be obtained as late as day 36 after injection of Daudi tumor cells, the data suggest that patients with minimal residual disease may be suitable for this therapeutic approach. The largest tumors that were effectively treated in this study were visible macroscopically, but only after dissection, and were thin discs <2 mm in diameter growing on the mesentery. Inasmuch as Daudi tumors were significantly more susceptible to this type of therapy than Raji tumors, we note that most B-cell lymphomas, like Daudi, have a normal p53 gene (27) and probably for this reason are relatively sensitive to radiation (28). This form of therapy is likely to benefit from multiple antibody injections in part because of the 3-day half-life of 111In. The advantage of two injections of 111In-LL1 was shown in Fig. 3 in which the first dose was given on day 36 after tumor inoculation. However, many additional studies are required to establish the optimal protocol for multiple injections. Although we elected to first give the MTD followed 2 weeks later by the highest possible second dose, it has been argued that fractionated dosing (in which multiple doses below the MTD are given) may be advantageous (29).

The antibodies used in these studies, LL1 and 1F5, both bind in large amounts to the cell targeted, and this is essential to kill cells with LEE emitters (9). With LL1, the high uptake is primarily due to continuous internalization and replacement of the cell surface molecules with newly synthesized molecules, because expression on the cell surface at any one time is relatively low. Although this was investigated most thoroughly with Raji cells (14), results with Daudi, RL, Ramos, and other B-lymphoma cells were similar (9). With Raji cells, 50,000 molecules are present on the cell surface and are replaced by newly synthesized molecules in an average of 40 minutes, so that ~10^7 antibody molecules are taken up per cell per 24 hours (14). Daudi cells were killed by 111In-LL1 somewhat more efficiently than Raji cells (9), but this difference can be attributed to the difference in radiation sensitivity that was noted above. Binding of 1F5 to RL cell was at a level of 3.0 × 10^5 molecules per cell after a 1-hour incubation at 37°C with a saturating concentration of 125I-1F5 (as described with Raji cells; ref. 26), and this increased 2.5-fold to 7.4 × 10^5 antibody molecules per cell after a 24-hour incubation. This level of uptake, therefore, is ~10-fold lower in magnitude than the uptake of LL1 but still at a relatively high level. Some of the 1F5 bound to RL cells accumulates in shed cytoplasmic fragments that were described recently (30), and this may have some effect on the therapy results, but this issue requires further investigation. In general, however, it should be noted that these quantitative antibody uptake results were all obtained in vitro, and antigen expression and processing may be quite different in vivo; indeed, we showed that catabolism of antibodies bound to the cell surface seems to be much faster in vivo than in vivo (19).

In as much as it is often stated that LEE emitters must be internalized by cells to effectively irradiate the nucleus (31–34), this issue should be further discussed. The results presented herein, together with our previous studies (9, 10), show that this is not the case and that internalization has little if any impact on the cytotoxic effect of LEE emitters delivered by antibodies. In this study, we showed that 1F5 conjugated to LEE emitters efficiently inhibited the growth of RL tumor xenografts, and 1F5 has been generally considered to be noninternalized (35). However, our recent experiments showed that, in fact, 1F5 is internalized and delivered to a noncatabolic intracellular site identified as the endocytic recycling compartment (26). Therefore, 1F5 cannot be used as an example of a non-internalizing antibody, but it should be noted that 1F5 internalization is at a rate much lower than that of LL1 and that 1F5 internalization by RL cells is at a much lower level than with other B-lymphoma cell lines (30). Therefore, the results with 1F5 show at least that the very high internalization rate seen with LL1 is not required to achieve effective therapy in vitro. In any case, the issue of antibody internalization was investigated more definitively, albeit in vitro, in previous experiments using the anti-HLA-DR antibody L243, because this is in fact a slowly internalizing antibody. [All antibodies bound to any cell line are gradually internalized over a period of days probably due to bulk turnover of cell membrane constituents (36, 37).] In thorough comparisons of L243 with LL1 and 1F5 in which the cumulative decays per cells were related to the fraction surviving, there was no evident impact of the internalization rate on the toxic effect (9, 10). The reasons for these apparently controversial results were discussed in some detail previously (10), but the key factors are the following: (a) the abundant Auger electrons of <5 keV are virtually irrelevant, because they cannot efficiently reach the nucleus from either the cell surface or the cytoplasm; (b) for 20 to 30 keV electrons, which are probably mainly responsible for killing, there is relatively little difference between cell surface and cytoplasmic decay, and the small difference that might be predicted is probably still an overestimate due to the fact that the nucleus is located close to the cell membrane as opposed to being in the center of the cell. If antibodies could deliver radionuclides to the nucleus, this would have a major impact, as we have discussed previously (38), but it is not likely that any of the antibodies we have used can do this to a significant extent.

These data, together with previous therapy results with disseminated B-cell lymphoma xenografts (7), suggest that antibody conjugates with LEE emitters may be more effective than conjugates with β-particle emitters for treatment of micrometastases. The previous study, with therapy at day 5 after i.v. inoculation of tumor cells, showed that 111In and 67Ga were markedly superior to 90Y. In the current study, with therapy at day 5 after s.c. inoculation of tumor cells, 111In was superior to 131I. However, it might be argued that neither of these was a fair comparison. The 131I label used herein (a conventional

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3 M.J. Mattes, unpublished data.
iodine label) is a nonresidualizing label; therefore, the residualizing $^{111}$In label had an advantage that was independent of the type of radiation emitted. In addition, $^{90}$Y is the least appropriate of the available $\beta$-particle emitters for therapy of micrometastases due to the very high energy of this $\beta$-particle. Furthermore, the specific activity of the $^{90}$Y conjugates used was relatively low in comparison with the $^{111}$In conjugates (although comparable with the specific activity of the $^{67}$Ga conjugates). A better $\beta$-particle emitter for this purpose is $^{177}$Lu, because this has a relatively low-energy $\beta$-particle and is a residualizing label. Therefore, we have recently tested $^{177}$Lu-LL1 for therapy in the disseminated lymphoma model: this was not substantially more effective than $^{90}$Y and remained less effective than the LEE emitters.

Considering that $\beta$-particle emitters are already established as effective therapeutic agents on anti-CD20 antibodies, one strategy would be to combine the two types of emissions, which in many respects are complementary. $\beta$-Particle emitters are expected to have a substantial advantage over LEE emitters in the therapy of macroscopic tumors (although there is no direct evidence supporting this hypothesis). Although the disadvantage of $\beta$-particle emitters is their greater nonspecific toxicity (6, 7, 10), which is due to cross-fire effects, cross-fire can also be an advantage in the killing of tumor cells that express low levels of the antigen targeted or that are in regions of the tumor not efficiently penetrated by antibodies. Behr et al. (34, 40) also compared LEE emitters and $\beta$-particle emitters using an antibody to colon carcinoma, anti-EGP-2 (39), to treat established s.c. xenografts of colon carcinoma. They did a comparison of $^{125}$I versus $^{131}$I and $^{111}$In versus $^{90}$Y. In both cases, at the MTD, the LEE emitter was a more effective therapeutic agent than the $\beta$-particle emitter. Thus, it seems possible that LEE emitters may be generally more effective than $\beta$-particle emitters at treating both large and small tumor burdens, but much further work is required to investigate this possibility.

Despite the successful clinical use of radiolabeled antibodies for therapy of B-cell lymphoma, there is little experimental animal data supporting this approach. We should emphasize that this may primarily reflect the limitations of the animal models available. Buchsbaum et al. (41), using an $^{131}$I anti-CD37 antibody, reported a small therapeutic effect that did not occur with a nonreactive antibody. However, the unlabeled antibody also had a substantial effect, and the combination of unlabeled antibody plus radiolabeled nonreactive antibody was not tested, leaving open the possibility that the effect of the radionuclide was nonspecific. Wei et al. (42) used $^{131}$I anti-CD22, $^{131}$I-CD19, and $^{131}$I-CD20 antibodies in a nude mouse xenograft model and obtained effective therapy with the first two. Because a nonreactive control antibody labeled similarly had very little effect, the therapy apparently was specific. The control mentioned above was not done, but this control seems less essential if the unlabeled antibody has no effect as was the case here (probably because the antibodies tested were all IgG1s). DeNardo et al. (43) described therapy with a $^{67}$Cu anti-MHC class II antibody. Thus, the prominent “gap” in experimental studies is that there is no evidence that anti-CD20 antibodies labeled with $^{131}$I or $^{90}$Y are effective agents, despite the fact that such conjugates have been effective in patients (44, 45). Our data with LEE emitters conjugated to an anti-CD20 antibody, presented herein, show greater therapeutic efficacy than has been shown previously with directly radiolabeled anti-CD20 antibodies in a mouse model. Recently, Press et al. (46) described effective therapy of s.c. lymphoma xenografts with $^{90}$Y using pretargeting methodology to target CD20. Our results also emphasize the value of experimental mouse models in understanding the processes that occur. For example, Fig. 4C shows that both unlabeled 1F5 and an $^{131}$I-labeled nonreactive antibody had some therapeutic effect alone, and a combination of the two appeared to be slightly more effective than either individual reagent, but this combination was still less potent than $^{131}$I-labeled 1F5. These type of control experiments, which cannot be done in patients, show that the effect of radiolabeled 1F5 is due to a combination of several mechanisms.

Two clinical trials have been done with LEE emitter-antibody conjugates, both of which used $^{125}$I. Anti-EGFR antibodies were tested in glioma patients (47), and antibody A33 was tested in colon carcinoma patients (48). Both of these studies were phase I/II and showed that high doses could be injected without reaching the MTD, up to 500 mCi in the work of Welt et al. (48). Major therapeutic effects were not observed in these studies. However, it is now realized that $^{125}$I was not a good choice for this purpose. The selection of $^{125}$I for these studies was based partly on the availability and convenience of this radionuclide but also partly on the idea that $^{125}$I is a particularly potent emitter of LEEs. This idea is based on the fact that $^{125}$I emits a large number of LEEs, >20 per decay. However, most of these electrons have <5 keV, so will not irradiate the nucleus unless they are delivered to the nucleus, which is unlikely to occur in substantial amounts, as noted above. From the published subcellular $S$ values (4), $^{111}$In and $^{67}$Ga would be expected to be comparable with $^{125}$I in potency, and there are three advantages of $^{111}$In or $^{67}$Ga over $^{125}$I. First, the long half-life of $^{125}$I, ~20 times longer than that of $^{111}$In or $^{67}$Ga, means that many more radionuclide atoms must be bound to deliver the same number of decays over a period of 1 to 2 weeks. Second, the radiometals, but not $^{125}$I, are residualizing radiolabels (49). [However, we note that there are novel radiolabeling methods that generate a residualizing form of iodine (50).] Third, $^{111}$In labeling can reach a specific activity that is at least 4-fold higher than that obtained with $^{125}$I. Although the specific activity is a function of the particular labeling methods that are used and may thus be improved by technical modifications, a reasonable goal is a 1:1 molar ratio of radionuclide-IgG, which is already reached for $^{125}$I; the specific activity of $^{125}$I conjugates is limited by the long half-life of this nuclide.

Killing by LEE emitters requires a high level of disintegrations per cell, meaning that specific activities must be high. This requirement may seem to be incompatible with the high protein doses of anti-CD20 antibodies that are normally used in patients (44, 45). However, this problem can potentially be circumvented by injecting the labeled antibody after therapy with unlabeled anti-CD20 antibody, because this
induces near-complete depletion of normal B-lymphocytes in the blood and lymphoid organs (51, 52) and because the need for a high antibody protein dose is due to abundant expression of the antigen on normal B-lymphocytes. In regard to the potential therapeutic use of radiolabeled anti-CD74 antibodies, the disadvantage of this antibody, in comparison with anti-CD20 antibodies, is its considerably greater reactivity with normal cells, including macrophage-lineage cells as well as B cells, and probably any other cells expressing MHC class II antigens. However, it should also be noted that extensive RAIT studies in patients have been done with radiolabeled Lym-1 (53), an antibody to HLA-DR, which has a very similar tissue distribution. In this case, also, it would be desirable to deplete antigen-positive normal cells before attempting therapy with LL1 conjugated to an LEE emitter; this could potentially be achieved by using an antibody to HLA-DR, which therefore would not compete with the radiolabeled LL1 that would be subsequently injected. Finally, it should be noted that although the antibody conjugates used have a high specific activity, it is not so high that immunoreactivity of the antibodies is affected (as shown in our experiments), because even at the highest specific activity, with $^{111}$In, only one third of the antibody molecules have a conjugated atom of $^{111}$In. In conclusion, these data suggest that LEE emitters conjugated to antibodies to CD20 or CD74 should be evaluated for therapy of B-cell lymphoma.

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