Single-cell Cytotoxicity with Radiolabeled Antibodies

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INTRODUCTION

We previously described the cytotoxicity for B-lymphoma cells of an Ab to CD74 (MHC II invariant chain, Ii) conjugated to Auger-electron-emitting radionuclides, including 125I, 111In, and 99mTc (1). This Ab is taken up in large amounts by target cells, approximately 10^7 Ab molecules/cell/day, because of constitutive rapid internalization of molecules at the cell surface and replacement by newly synthesized molecules. Abs internalized from the cell surface are delivered to lysosomes and promptly degraded, together with Ii. If residualizing radionuclides are used, they accumulate within cells to high levels. Residualizing labels are defined as those that generate catabolic products that are unable to efficiently cross membranes and therefore are trapped within lysosomes. The level of radioactivity reached in these experiments was >50 cpm/cell, and 100% of the target cells were killed (5 × 10^5 cells total). Dosimetry calculations indicated that the radiation dose delivered was consistent with the cytotoxicity observed.

The current study was intended to extend these results in two ways: to test other cell lines expressing Ii, including adherent target cells; and to test other Abs reacting with B-cell lymphomas. Melanoma cells and many carcinoma cells can be induced to express high levels of both mature MHC class II antigen and the invariant chain by IFN-γ. Anti-CD74 uptake by induced SK-MEL-37 melanoma cells was comparable with the uptake by Raji cells (2). However, the very different cellular morphology is likely to have a significant impact on killing by internalized radionuclides. More specifically, the spreading of the cells on plastic would tend to decrease the proximity of nucleus and cytoplasm. Therefore, we have tested cytotoxicity for these adherent cell lines, as well as for other B-cell lymphomas, to establish the generality of the results.

Whereas the massive intracellular uptake of anti-CD74 is not matched by any other Ab to our knowledge, some other Abs can bind to the cell surface in similar amounts. For example, the number of mature MHC class II molecules on Raji B-lymphoma cells is approximately 3 × 10^6 (2). (Note that the mature MHC class II molecules lack the invariant chain, which must be removed before binding of peptide antigens.) This level of expression suggests that some cytotoxicity might be obtained with anti-MHC class II, although perhaps not as strong as with anti-CD74. A basic difference between anti-CD74 and immature MHC class II is in their subcellular localization. Thus, whereas the former is delivered to lysosomes, the latter remains primarily on the cell surface. According to dosimetry calculations, isotopes in the cytoplasm are expected to be more potent than isotopes on the cell surface, but the difference is relatively small for the radionuclides used in these experiments. For example, the advantage of internalization for 125I and 111In is 53

1 The abbreviations used are: MHC, major histocompatibility complex; Ab, antibody; DTPA, diethylenetriaminepentaacetic acid; con A, concanavalin A; TCA, trichloroacetic acid.

ABSTRACT

Previous studies demonstrated the effective, antigen-specific killing of Raji B-lymphoma cells in vitro by radiolabeled anti-CD74, attributable largely to the high level of uptake, of approximately 10^7 antibody (Ab) molecules/cell/day. This Ab is rapidly delivered to lysosomes for catabolism, so the radionuclide delivered accumulates primarily in lysosomes. In this study, we have tested Abs that bind to the same target cells in similar amounts, but remain primarily on the cell surface, to compare the potency of radioactivity delivered to the cell surface versus the cytoplasm. The Abs tested were anti-major histocompatibility complex class II and anti-CD20. 111In-labeled conjugates made with these two Abs killed cells very effectively and specifically, with 100% kill of sample of 5 × 10^6 cells. Because these Abs remained primarily on the cell surface, it would be predicted that residualizing radionuclides, which are trapped in lysosomes after Ab catabolism, would not be required, and this was observed, i.e., these two Abs were effective when labeled with either 125I or 131I, using conventional iodination, as well as with the residualizing label 111In-labeled DTPA. These results are in contrast to results obtained with anti-CD74, which required a residualizing radiolabel for effectiveness. The uptake of these radionuclides, in cpm/cell, was monitored, and this allowed estimation of the radiation dose delivered; the cytotoxicity observed was consistent with the estimated radiation dose delivered. To establish the generality of the results, we also demonstrated that 111In-labeled anti-CD74 effectively killed three other B-lymphoma cell lines, in addition to Raji and the adherent melanoma cell line SK-MEL-37. By using more potent radionuclides or conjugates of higher specific activity, this approach might be effective with other, lower density antigens.

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approximately 3–5 days until the cells had multiplied 16-fold or until day 21. The fraction of cells killed was calculated from the growth curves, without considering possible division delay induced by irradiation.

Cytotoxicity of Adherent Cells. SK-MEL-37 melanoma cells were preincubated for 2 days with human IFN-γ (IFN-γ-b; Actimmune; Genentech, South San Francisco, CA) at 250 units/ml, and IFN-γ at this concentration was included in the medium used throughout the assay. After trypsinization, 0.15 ml of a 0.1% suspension (packed cell v/v) was plated in wells of 96-well plates and incubated overnight. After aspiration of the medium, 0.2 ml of medium containing radiolabeled Ab was added. Serial dilutions of the Abs were used, each tested in triplicate, and the incubation was for 48 h. The control wells contained excess unlabeled Ab at 62.5 μg/ml to inhibit specific Ab binding and thereby to indicate the level of nonspecific cytotoxicity. Some wells were used to measure uptake of radioactivity, some were used for cell counts after trypsinization, and some were used for a clonogenic assay. To measure uptake, cells were washed three times with tissue culture medium and then harvested with 50 μl 2.0 M NaOH, which was collected with a cotton swab. For the clonogenic assay, cells from a single well were trypsinized and suspended in 6.7 ml medium. Serial 4-fold dilutions were prepared, and 5 ml of each dilution were plated in T30 flasks. After 12–15 days of growth, colonies of >50 cells were counted in those flasks that contained 50–200 colonies. Before counting, colonies were fixed for 10 min with 5.0% glutaraldehyde in 0.1 M sodium cacodylate HCl (pH 7.2), stained for 10 min with 0.5% methylene blue in 25% ethanol, and washed thoroughly with water. Irradiated cells grew more slowly than control cells, which can be attributed to radiation-induced growth delay (10) or to other toxic effects of the radiation. Therefore, compared with the control cells, the treated cells were allowed 2–3 more days to grow before counting colonies. In practice, this allowed all of the healthy colonies to reach the countable size of ≥50 cells. The cloning efficiency of control cells was approximately 60%.

Monitoring Uptake of Radioactivity. Uptake of cpm was determined in experiments identical to the cytotoxicity experiments. At various times, cells were collected, pelleted, and washed three times with 5 ml tissue culture medium, and the radioactivity was determined. Routinely, 1/20 of the total sample was counted because otherwise the cpm were too high to be counted accurately (>10⁶ cpm). Cell counts were also determined at each time point, although, for reasons discussed below, the data are generally expressed in terms of the initial cell count. The number of Ab molecules bound/cell was calculated from the cpm bound and the specific activity, and was corrected for the fraction of cpm in the stock Ab preparation that was not associated with intact IgG (always <10%). For radiation dosimetry, the area under the curve of bound cpm versus time was calculated, which provided the cumulative counts. Cumulative disintegrations were calculated from the gamma counter efficiencies, which were 70.9% for ¹¹¹In and 76.5% for ¹²⁵I. Cellular S-values for Raji-sized cells (diameter, 16 μm; nuclear diameter, 12 μm) were from Goddu et al. (3).

Elongation of Bound Radiolabeled Ab by Excess Unlabeled Ab. Experiments were set up like the cytotoxicity experiments, with cells in 24-well plates. ¹²⁵I-labeled L243 was in-
RESULTS

Killing of Various Target Cells with \( ^{111}\text{In}\)-labeled Anti-CD74. Previous cytotoxicity experiments were performed only with the Raji B-lymphoma cell line (1). To establish the generality of the results, similar experiments were performed with three other B-cell lymphomas, Ramos, Daudi, and RL. Ramos and Daudi, like Raji, are Burkitt lymphoma cell lines, whereas RL is derived from a diffuse non-Hodgkin lymphoma. Daudi is the only one of these four lymphoma cell lines that has a normal \( p53 \) gene (12, 13), which might be expected to increase its sensitivity to radiation (14). Cytotoxicity experiments were performed similarly to those described previously, with serial dilutions of the radiolabeled Ab starting at 90 \( \mu \text{Ci/ml} \). The results, shown in Fig. 1, were generally similar to the results described with Raji cells, with high levels of antigen-specific cytotoxicity with all of the cell lines. With three of the four cell lines, 100% killing was obtained (> approximately 6 logs) at the highest Ab concentration tested, whereas the killing of RL cells was slightly less. Daudi was markedly more susceptible to killing than the other three cell lines, with both antigen-specific and nonspecific killing. In the assay of nonspecific killing, a nonreactive Ab at 90 \( \mu \text{Ci/ml} \) killed 85–96% of Daudi cells, whereas killing of the other three cell lines was only 30–50%. By comparison with previous experiments in which higher \( \mu \text{Ci} \) concentrations were tested in the killing of Raji (15), Daudi was approximately 3-fold more sensitive to nonspecific killing. In specific killing with \( ^{111}\text{In}\)-labeled LL1, Daudi was also 2–3-fold more sensitive, as shown in Fig. 1. Also, unlike the other target cells, Daudi cells did not gradually become very large before lysis, as described previously for Raji (1), but instead lysed more rapidly. Both the lack of cell enlargement and the susceptibility to killing of Daudi cells are likely to be related to the presence of wild-type \( p53 \), which plays a role in the induction of apoptosis (16), but additional studies are required to confirm this possibility.

An adherent cell line, SK-MEL-37, which has a level of Ab uptake similar to that of the B-cell lymphomas (2), was also tested as a target cell. These cells were adherent to plastic throughout the assay, which was considered to be a potentially important difference from the B-cell lymphomas. As shown in Fig. 2, strong, antigen-specific killing was observed. With the highest Ab concentration tested, 10 \( \mu \text{g/ml} \), the concentration of radioactivity was 159 \( \mu \text{Ci/ml} \). The nonspecific cytotoxicity (O) was determined by inclusion of excess unlabeled Ab. The results shown are representative of six experiments, using slightly different Ab concentrations and specific activities.

![Fig. 1 Cytotoxicity of four lymphoma cells with \( ^{111}\text{In}\)-labeled LL1.](Image 1)

![Fig. 2 Cytotoxicity of SK-MEL-37 melanoma cells with \( ^{111}\text{In}\)-labeled LL1.](Image 2)
Killing Raji Cells with Other Abs Conjugated to 111 In, and Uptake of the Radiolabel. Two other Abs, reacting with relatively high-density antigens, were labeled with 111 In and tested for their ability to kill Raji cells. Antimature MHC class II (Ab L243) and anti-CD20 both killed cells very effectively (Fig. 3). In comparison with the previous CD74 experiments, L243 was comparable in potency, whereas CD20 was somewhat less potent on the basis of the dose-response curves. At the highest concentration tested, CD20 killed slightly 100% of the target cells. For example, in the experiment shown in Fig. 3, the fraction surviving with 1F5 at 90 μCi/ml was 2.99 × 10^-7, which corresponds to approximately 1–2 cells surviving of the initial 5 × 10^5 cells. Nonreactive Abs labeled in the same way had no significant cytotoxic effect at the concentrations used, as shown in Fig. 3C, demonstrating that the cytotoxicity was antigen-specific.

For both Abs L243 and 1F5, unlabeled Ab used at the same protein concentration was tested to demonstrate that the cytotoxicity was attributable to the radiation delivered. The Ab concentration was 4–5 mg/ml, which is a near-saturating concentration. Results with unlabeled L243 are shown in Fig. 3A. This Ab appeared to have a transient effect at day 2, which was probably because of the fact that the Ab causes marked clumping of the cells, but by day 5 the cells had multiplied essentially as much as untreated cells. In a similar experiment with unlabeled 1F5, the Ab had no detectable effect on cell growth (Fig. 3B).

Uptake of cpm was determined in similar experiments. As shown in Fig. 4, the uptake of both Abs was very high. The peak uptake with L243 was 47 cpm/cell, and the peak uptake with 1F5 was 29 cpm/cell. Although we determined the cell count at each time point in all of the experiments, the data are presented in terms of the initial number of cells plated/well. The reason for this is that the cells start to die as early as day 2, and therefore use of the actual viable cell number results in artifactual high values of cpm/cell at later time points. For example, in the experiment shown in Fig. 4, L243 killed approximately 70% of the initial cells at day 2, which resulted in a value of >150

Fig. 3  Cytotoxicity of Raji cells with 111 In-labeled Abs L243 (A) or 1F5 (B). Cells were incubated for 2 days with radiolabeled Ab at a starting concentration of 90 μCi/ml (●), 30 μCi/ml (□), 10 μCi/ml (▲), or 3.3 μCi/ml (▼). The growth rate of control, untreated cells is also shown (dotted line without symbols). Also shown is the effect of unlabeled Ab at a concentration equal to that used for the highest concentration of radioactivity (○); this was 4.7 μg/ml for L243 and 3.7 μg/ml for 1F5. Data shown are cell counts obtained at various times and are representative of two or three experiments with each Ab, each done in duplicate. Cells treated with the highest concentration of L243 were 100% killed, because no viable cells were detected after day 6, and the growth of a single viable cell would be readily detected in 22 days. Panel C shows the calculated surviving fraction as a function of initial μCi/ml for the same experiments, with L243 (●), 1F5 (□), and a nonreactive control Ab labeled similarly (▲). Because the specific activities and immunoreactivities of the Abs were not identical (although they were similar), this graph does not allow a precise comparison of the Abs. The highest concentration of L243, 90 μCi/ml, killed 100% of the cells and therefore cannot be plotted on the logarithmic Y-axis.

Fig. 4  Uptake of 111 In-labeled L243 (●) or 1F5 (□) by Raji cells. Both Abs were used at 56 μCi/ml, which was 2–2.4 μg/ml. The incubation of Ab with cells was for 2 days, followed by a 14.3-fold dilution. This treatment resulted in a surviving fraction of 0 with L243 and 7.76 × 10^-6 with 1F5. Panel A shows the cpm/cell determined at various times. This was calculated based on the initial cell number rather than the actual cell number at each time point, for reasons discussed in the text. Panel B shows the Ab molecule-equivalents bound/cell, which is expressed in this way because some of the cpm were from Ab molecules that had been catabolized. Results shown are representative of two similar experiments.
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With 1F5, however, the high level of Ab uptake/cell, peaking at approximately $5 \times 10^6$ Ab molecules/cell at day 2, is not so readily explained. The number of CD20 sites/cell on Raji cells was reported to be $3.6 \times 10^5$ (7), which is similar to the value reported for another B-lymphoma cell line, Daudi (6). We redetermined the sites/cell at saturation and found that there were $2.4 \times 10^5$ sites/cell, which is consistent with the previous reports. Thus, the number of $^{111}$In-labeled Ab molecules bound/ cell at 48 h, as shown in Fig. 4, was approximately 20-fold higher than the number of sites/cell. The increase in cell size because of lethal irradiation, as noted above, can account for only a 2–3-fold increase, so a 10-fold increase remains to be accounted for. Another factor, as with L243, is the turnover of the antigen (and any Ab bound to it). However, CD20 is considered to turn over slowly, as indicated by low rates of catabolism of bound Ab. Press et al. (6) reported that there was no significant catabolism of a CD20 Ab by Daudi cells. We described significant levels of catabolism using the Ramos cell line, but this was still at a relatively low level (17). The impact of this factor can be readily investigated by comparing residualizing with nonresidualizing radiolabels. A convenient nonresidualizing label is a conventional chloramine-T iodine label, because iodotyrosine rapidly leaves the cell after it is generated in lysosomes (discussed in Ref. 18). Results of such a comparison with anti-CD20 demonstrated that the advantage of a residualizing label is modest, <2-fold. Therefore, the high level of binding of 1F5, much higher than the number of sites/cell, remains to be explained and is discussed further below.

Killing Cells with L243 or 1F5 Conjugated with $^{131}$I or $^{125}$I, and Uptake of $^{125}$I. Because both of these Abs are internalized at relatively low rates, the majority of bound Ab is expected to be at the cell surface, at least for the first 2–3 days of incubation with Ab. The implication of this statement is that the cells are killed primarily by radiation emitted from the cell surface. This represents a major difference from the situation with LL1, because this Ab is localized primarily to lysosomes. Because cytoplasmic radionuclides are expected to be somewhat more cytotoxic than radionuclides on the cell surface, as noted in the “Introduction,” it seemed important to confirm further that radionuclides on the cell surface were responsible for cell killing. Accordingly, experiments were performed with conventional iodine labels, both $^{131}$I and $^{125}$I. These labels, as noted above, rapidly leave the cell after catabolism, so are not trapped to a significant extent in lysosomes. Therefore, they remain localized in target cells primarily at the cell surface.

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Fig. 6 demonstrates effective cytotoxicity obtained with both $^{131}$I and $^{125}$I labels. Both radiolabels were tested at the highest concentration that produces little if any nonspecific killing, as determined previously (1, 15). The $^{131}$I-labeled conjugate was tested at a starting concentration of 16 $\mu$Ci/ml, whereas the $^{125}$I-labeled conjugate was tested at concentrations up to 138 $\mu$Ci/ml. With both isotopes, L243 was considerably more potent than 1F5, but 1F5 still had high levels of antigen-specific cytotoxicity. For example, in Fig. 6B, the cytotoxicity

4 Unpublished observations.
5 Unpublished observations.
uptake of $^{131}$I-labeled Abs is expected to be very similar, be-
determined with both Abs, and results are shown in Fig. 7. The
fraction is $1 \times 10^{-6}$, a reasonable estimate considering that the number
of starting cells/sample was approximately $5 \times 10^{5}$. The data with
$^{125}$I-labeled LL1 were taken from a previous publication (1) and is
included here for the purpose of comparison.

Fig. 6 Cytotoxicity for Raji cells of chloramine-T-labeled L243 (●),
If5 (■), LL1 (▼), or a nonreactive control Ab (▲). The radionuclide
used was either $^{125}$I (A) or $^{111}$In (B). Samples having a surviving fraction
of 0 cannot be plotted on the log axis, so are plotted as if the surviving
fraction is $1 \times 10^{-6}$, a reasonable estimate considering that the number
of starting cells/sample was approximately $5 \times 10^{5}$. The data with
$^{125}$I-labeled LL1 were taken from a previous publication (1) and is
included here for the purpose of comparison.

from $^{131}$I-labeled If5 may seem weak in comparison with L243,
yet it produced a cell kill of >3 logs. Fig. 6 also includes data
with LL1 for comparison, as well as data with a nonreactive
control Ab. With LL1, the labeled conjugate is rapidly catalo-
gized and released from the cells, resulting in little accumulation
and consequently little cytotoxicity, as shown.

Using an $^{125}$I label, the uptake of radioactivity was also
determined with both Abs, and results are shown in Fig. 7. The
uptake of $^{131}$I-labeled Abs is expected to be very similar, be-
cause the specific activities and the immunoreactivities with
both isotopes were similar. These experiments were set up
exactly like the cytotoxicity experiments, using the minimum
Ab concentration that kills nearly 100% of the cells. This
concentration was 16 μCi/ml for L243 and 138 μCi/ml for If5,
so 8.6-fold higher for If5. As shown, high levels of uptake were
observed with both Abs, with a peak uptake of approximately 20
cpm/cell at 2 days.

Radiation Dosimetry. From the area under the curve on
the plot of cpm versus time, the cumulative counts were deter-
mined for the uptake experiments described above. The cumu-
lative dpm were then calculated from the gamma counter effi-
ciency. Using the published S-values for $^{125}$I present on the cell
surface, $1.15 \times 10^{-4}$ Gy/Bq/sec, for a cell the size of Raji, the
total radiation dose delivered was 1386 cGy with $^{125}$I-labeled
L243 and 1530 cGy with $^{125}$I-labeled If5. These values are
presented in Table 1, which also lists the calculated cGy dose
delivered in 2 days. We have not determined the time of func-
tional cell death, but it is likely that most of the dose delivered
at later time points was redundant, because the cells were
already dead. Similar values are also shown for $^{111}$In-labeled
conjugates. With the $^{111}$In label, the situation is complicated by
the fact that some of the bound Ab is internalized and catalo-
gized, and the catabolites are retained within lysosomes. Thus,
the label is not entirely on the cell surface, and it may be
appropriate to use the cytoplasmic S-value for some fraction of
the radiolabel. However, we know from previous studies that the
great majority of the bound Ab remains on the cell surface with
these two Abs (see below, also), so use of the cell surface
S-value is appropriate to estimate the radiation dose delivered.
We have not attempted to correct these values of radiation dose
for the gradual increase in the size of the cells resulting from
lethal irradiation, which was described above. This Table is
intended only to provide an estimate of the radiation dose
delivered in these experiments and does not allow a precise
comparison between Abs and radionuclides, because the uptake
naturally depends on the Ab concentration used. For $^{131}$I, as-
suming the same uptake as with $^{125}$I, the dose delivered would
be only slightly lower than with $^{125}$I, because the S-value is
$1.06 \times 10^{-4}$ Gy/Bq. Radiobiological parameters for Raji,
using external X-irradiation, was determined previously (1). The
$D_{0}$ was 90 cGy, and the extrapolation number $\bar{n}$ (10) was 1.31.
Therefore, the expected killing is consistent with the cytotoxic-
ity observed. The expected dose for a kill of 6 logs is 1268 cGy.

Ab L243 Bound to Raji Cells Remains Predominantly
on the Cell Surface. Although it seems clear that the great
majority of the bound iodine must be present on the cell surface
at all times because catabolites are released rapidly from the
cell, we demonstrated this directly by competitive elution with
excess unlabeled Ab, using L243. This method, which was
described and characterized previously (11), is based on the fact
that Abs bound to the cell surface frequently wobble (releasing
one of the antigen molecules), although they are predominantly
bivalently bound. For this reason, such Abs can be competi-
tively replaced by unlabeled Ab that is present at a high con-
centration. As shown in Fig. 8, the great majority of the bound
L243 Ab (80–90%) was eluted with excess unlabeled Ab, and
this fraction was the same whether the initial Ab incubation was
for 2, 22, or 47 h. These data demonstrate that the Ab remains
on the cell surface and does not accumulate in any intracellular
compartment to a substantial level. Because elution in these
experiments requires 24 h for completion, the possibility re-
 mains that the bound Ab recycles between the cell surface and
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effectively kill single cells, but this conclusion was based on a
Abs either. It is very unlikely that crossfire would be significant with these
L243 and 1F5 bound at similar or lower amounts to the cells, it
ments, was 7.8 
conjugates. The actual fraction surviving, determined in these experi-

DISCUSSION

we can conclude that the bound Ab spends at least some of the time
an intracellular compartment, such as early endosomes, but we
at the cell surface.

There are four major conclusions of this study: a) target
cells in addition to Raji (which were tested previously) were
killed effectively by anti-CD74; Susceptible targets included
three other B-cell lymphomas and the adherent melanoma SK-
MEL-37; b) Abs to antigens other than CD74, linked to either
Auger electron emitters or 
particle emitters, effectively killed target cells; c) Abs that remain primarily on the cell surface were effective cytotoxic agents; Internalization of bound radio-
nuclides into lysosomes is not required; and d) Abs that remain on the cell surface, it was predictably not necessary to
use residualizing radiotools to obtain strong cell killing. Al-
though it remains likely that internalization provides some ad-
vantage, as predicted by the calculated S-values, the expected
advantage is only slightly <2-fold for the radionuclides used
here. On the other hand, there is likely to be an advantage of cell
surface-bound Abs, in that the Abs can saturate the antigenic
sites rapidly in a few hours. With anti-CD74, in contrast, the
level of binding at 2–4 h is very low, and 24 h are required for the
maximum level of uptake to occur. Therefore, cell surface-
bound Abs such as L243 deliver higher radiation doses during the first 24 h, which probably explains why cytotoxicity with
L243 was observed earlier than with LL1. We investigated
previously (1, 15) the possibility that radiation crossfire (cell to
cell) is a significant factor in experiments of this type by plating
cells at different concentrations. With both 
- and 
- labeled LL1, crossfire was not a significant factor. Because
L243 and 1F5 bound at similar or lower amounts to the cells, it
is very unlikely that crossfire would be significant with these Abs either.

Earlier work suggested that radiolabeled Abs would not
effectively kill single cells, but this conclusion was based on a
limited body of experimental data, much of which we discussed
previously (1). Warters et al. (19) first attempted to kill cells
with 
bound to the cell surface. They used 
-labeled con A, which is expected to bind to a very large number of sites/cell, but only weak killing was observed. The maximum killing
obtained was approximately 90%. But there were several problems
associated with the use of con A. First, the bound con A
was very rapidly catabolized by the cells, which made it neces-
sary to perform the binding incubation for 36 h at 4°C to
maintain the con A on the cell surface. Second, the unlabeled
con A was cytotoxic for the cells, necessitating the use of trypsinized, monovalent con A. The avidity of this monovalent
ligand is unlikely to be as high as that of bivalent Abs, which
bind essentially irreversibly (11, 20). In any case, it should be
noted that the 
(dose required for 63% kill in the linear part of the semilog dose-response curve) for 
-labeled con A was
12,600 disintegrations/cell with CHO cells, which is in the same
range as the values we found with Ab LL1. The 
for 
-labeled IMP-R2-LL1 was approximately 24,900 with Raji cells,
which is in the same range as the values we found with Ab LL1. The
- of the 
-labeled IMP-R2-LL1 was approximately 24,900 with Raji cells,
and 0 for the other conjugates.

The disintegrations/cell, using the initial number of cells plated.
This value was used instead of the actual number of cells at each time
point, for reasons discussed in the text.

The last time point was day 4 for 
-labeled L243, day 6 for
-labeled 1F5, and day 5 for both 
-labeled conjugates.

Table 1

<table>
<thead>
<tr>
<th>Radioconjugate</th>
<th>Day 2</th>
<th>Last time point</th>
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<tbody>
<tr>
<td></td>
<td>Disintegrations</td>
<td>cGy</td>
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<tr>
<td>125 I-labeled L243</td>
<td>71,386</td>
<td>821</td>
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<tr>
<td>125 I-labeled 1F5</td>
<td>58,843</td>
<td>679</td>
</tr>
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</tr>
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* The initial Ab concentration was selected to be the minimum dose
that produced near 100% kill. This was 16 
/ml for 
-labeled L243, 138 
/ml for 
-labeled 1F5, and 56 
/ml for both 
-labeled conjugates. The actual fraction surviving, determined in these experi-
ments, was 7.8 × 10⁻⁶ for 
-labeled 1F5, and 0 for the other conjugates.

* The disintegrations/cell, using the initial number of cells plated.
This value was used instead of the actual number of cells at each time
point, for reasons discussed in the text.

* The last time point was day 4 for 
-labeled L243, day 6 for
-labeled 1F5, and day 5 for both 
-labeled conjugates.

Fig. 8

125 I-labeled L243 remains on the cell surface during a 2-day prolonged incubation. Raji cells were incubated with 
-labeled L243 for varying periods up to 47 h. At the times indicated, cells were
collected, pelleted, washed twice, and then incubated with 1.0 ml
medium either with (black bars) or without (cross-hatched bars) a large excess of unlabeled Ab. After overnight incubation to allow replacement of the bound labeled Ab by the unlabeled Ab, the distribution of the radiolabel was analyzed. The percentage of the total cpm that was
cell-bound, intact in the supernatant (TCA-precipitable), and degraded in the supernatant (TCA-nonprecipitable) was determined. Results
shown are means and SD of duplicates and are representative of two experiments. The excess unlabeled Ab induced the release from the cell
of most of the bound labeled Ab, and this was independent of the length
of incubation.
that time was redundant. If this were the case, the true $D_0$ would be much less than our estimate. In general, we consider the results of Warters et al. (19) to be consistent with our data.

Another key study was by Lindmo et al. (21), who attempted to kill melanoma cells with an $^{131}$I-labeled Ab that reacts with a high density antigen on the cell surface. These authors showed that strong killing was obtained only if Ab-coated cells were stored frozen for many weeks to allow radiation damage to accumulate. This result, then, emphasizes the low level of cytotoxicity obtained. In general, the relatively weak cytotoxicity that has rarely been reported with radiolabeled Abs has been attributed to unusual properties of the particular Abs, such as transport to the nucleus (22, 23) or accumulation in macropinosomes (24). Cytotoxicity with $^{131}$I-labeled anti-CD20 was recently reported by Johnson and Press (25). These investigators intentionally pelleted the target cells to increase crossfire from adjacent cells, so their results may or may not demonstrate single-cell kill. We note that $\alpha$-particle emitters are exceptions to many of the above statements. This type of radiation can efficiently kill single cells (26); however, the available isotopes have short half-lives of $\approx 7$ h, which seem unsuitable for treatment of solid tumors where substantial time is required for tumor penetration. $\alpha$-Particle emitters also appear to be very toxic, considering that only 20 $\mu$Ci of $^{212}$Pb-labeled Ab can be administered to a mouse (27).

From the cellular $S$-values published by Goddu et al. (3), we can calculate the number of disintegrations on the cell surface required to produce a defined level of cell kill. For Raji cells, the radiation parameters, determined from Cs$^{137}$ irradiation experiments, were $D_0 = 90$ and $\bar{n} = 1.31$ (1), so the dose required for a kill of 6 logs is approximately 1270 cGy. For a cell the size of Raji ($R_c = 8 \, \mu$m; $R_H = 6 \, \mu$m), this would require approximately 110,000 disintegrations of $^{125}$I, 120,000 disintegrations of $^{131}$I, and 164,000 disintegrations of $^{111}$In. From our data, the amount of radioactivity delivered/cell reached these levels, and the cytotoxicity observed was generally consistent with the calculated radiation dose. However, as noted above, the time course of irradiation is an important factor that has not been incorporated into the calculations.

Although the ability to kill single cells is clearly critical for therapy of micrometastases, its importance for therapy of solid tumors is uncertain. Indeed, radiolabeled Abs used for radioimmunotherapy, both experimentally and clinically, are very rarely even tested for in vitro cytotoxicity. This is partly because crossfire is a major factor in the killing of cells within solid tumors, whereas it does not apply in the killing of single cells (28). In vivo experiments, both in animal models and in patients, are required to ascertain the relevance of single-cell kill to therapy. The results described herein do demonstrate, however, that two of the Abs that have been most effective in clinical studies of radioimmunotherapy, anti-CD20 (29) and anti-MHC class II (30), are both in fact able to effectively kill single cells. This suggests that the importance of single-cell kill may be underestimated. At the doses used in patients, initial Ab concentrations are high enough to produce substantial single-cell kill, according to our in vitro data. For example, with a dose of approximately 60 mCi of $^{131}$I-labeled Lym-1, as used by Denardo et al. (30), the peak concentration in interstitial fluid will be approximately 6 $\mu$Ci/ml, which from our in vitro data would produce a high level of cell kill. This is also true for the myeloma cell line DOXO-8, which was used by Press et al. (31). We note that the anti-MHC class II Ab used herein, L243, is not allele specific, unlike Lym-1 (32), so would be expected to have more consistent reactivity with diverse B-cell lymphomas.

An enigma regarding anti-CD20 binding is that the uptake of Ab is much higher than the number of sites/cell by a factor of approximately 20. There are two known factors that contribute to increasing the Ab uptake. First, there is some turnover of the antigen, which provides some increase in the Ab molecule-equivalents binding/cell. Second, once radiation damage occurs, the cells increase markedly in size, which naturally results in an increase in the Ab molecules bound/cell. However, these factors can explain only a 3–4-fold increase in Ab sites/cell, so a further explanation is required. Two possibilities are apparent. First, the level of antigen may be up-regulated as a result of Ab binding. Secondly, there may be a pool of intracellular antigen that equilibrates with the cell surface and is gradually bound by Ab. Which of these possibilities is correct, if either, is currently under investigation.

Although the 98% kill of the adherent target cells, SK-MEL-37, was substantial, it was not as complete as the killing of B-lymphoma cells, which was 100%. This is likely to be attributable at least partially to the shape of the cell. Although we have not attempted to perform dosimetry calculations for cells adherent to plastic, which would be quite complex, it is clear that the flattened shape of the cell will tend to separate radioisotopes in the cytoplasm. Moreover, the rim of cytoplasm will be quite thin above and below the nucleus. Thus, the radiation dose delivered to the nucleus will be considerably less than with round lymphoma cells. In addition, the SK-MEL-37 cells may be less sensitive to radiation than B-lymphoma cells. We would also like to suggest another possible contributory factor, though speculative. When the SK-MEL-37 cells form a monolayer, the cells are always most dense along the edges of the wells, and it seems possible that a few cells may be buried beneath other cells at the edges and therefore inaccessible to Ab.

The major advantage of Auger electron emitters over $\beta$-particle emitters appears to be their much lower level of nonspecific cytotoxicity (15). This was demonstrated in vitro (15) and has also recently been shown in vivo in a SCID mouse xenograft model. In regard to single-cell kill alone, $^{131}$I was more potent than either $^{111}$In or $^{125}$I on the basis of the $\mu$Ci/ml required for a particular level of kill, and this was true for all of the three Abs tested. The potency of $^{131}$I can be at least partially attributed to its significant nonspecific toxicity, which will enhance the effect of specific Ab binding. The higher energy $\beta$-particles of $^{90}$Y (relative to $^{131}$I) are expected to be somewhat less potent at single-cell kill, and this was demonstrated in vitro with Ab LL1 (15).

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6 R. Ochakovskaya, L. Osorio, D. M. Goldenberg, and M. J. Mattes. Therapy of disseminated B-cell lymphoma xenografts in SCID mice with an anti-CD74 antibody conjugated with $^{111}$In, $^{67}$Ga, or $^{90}$Y, submitted for publication.
The three antigens targeted in this study are clinically important. However, the significance of these results will be amplified if the approach can be applied to other antigens that are not present at such a high density. This can potentially be achieved by using more potent radionuclides or higher specific activity. It should be emphasized that the radionuclides we have tested were selected only because of availability and that many more potent radionuclides (based on theoretical S-values) could be selected. There are at least four radionuclides that are expected to be at least 3-fold more potent than $^{111}$In when delivered to the cell surface, having suitable half-lives of 1.7–15.4 days. These are $^{153}$Sm, $^{191}$Os, $^{195m}$Pt, and $^{195m}$Hg. $^{191}$Os would also be potent, because it decays rapidly to $^{191}$Os. On the basis of the analysis of Sastry et al. (33), $^{119}$Sb and $^{119m}$Te are also expected to be potent (although cellular S-values have not been published for these isotopes). $^{195m}$Pt, the most potent of the group, is expected to be 9.9-fold stronger than $^{111}$In. An isotope like $^{153}$Sm is attractive because it emits both abundant conversion electrons and moderate-energy $\beta$-particles. None of these radionuclides are currently available carrier-free, and only $^{153}$Sm has been conjugated to Abs with high stability (34). Large increases in specific activity, using currently available isotopes, are also possible. Conjugation of a single $^{111}$In atom/Ab would yield a specific activity of approximately 240 mCi/mg, which is 6-fold higher than the highest specific activity we have used in these experiments. Furthermore, it has been demonstrated that some Abs can be conjugated with five chelates without impairing immunoreactivity (35). In summary, there are realistic approaches by which much lower-density antigens might provide effective targets.

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Single-cell Cytotoxicity with Radiolabeled Antibodies

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