Comparisons between Two Monoclonal Antibodies That Bind to the Same Antigen but Have Differing Affinities: Uptake Kinetics and ¹²⁵I-Antibody Therapy Efficacy in Multicell Spheroids

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ABSTRACT

It has been predicted that low affinity antibodies (Abs) should penetrate into tumors more readily than high affinity Abs. However, the absolute uptake and residence time of a high affinity Ab may be better. It is, therefore, not clear whether a high affinity Ab would have a therapeutic advantage. This is particularly relevant with ¹²⁵I radioimmunotherapy, where targeting of every cell is important.

This study compared the uptake kinetics and toxicity in multicell spheroids of two murine monoclonal Abs labeled with ¹²⁵I. 17-1A was produced by immunization with a human colon cancer cell line and has an affinity of 5.15 x 10⁷ M⁻¹. 323/A3 was produced by immunization with a human breast cancer cell line and has an affinity of 1.87 x 10⁹ M⁻¹.

Binding of both Abs to LS174T spheroids was similar at 4°C, but binding of 17-1A was 8-10-fold less than that of 323/A3 at 37°C. Despite this difference, the toxicity of ¹²⁵I-17-1A in spheroids after 7 days of incubation was similar to that of ¹²⁵I-323/A3. Autoradiography showed that 17-1A penetrated the spheroids much more deeply and evenly than did 323/A3. It appears that much of the radiation dose to spheroids treated with 323/A3 was wasted because of the uneven Ab distribution.

This study demonstrates the potential advantage of using Abs of lower affinity for ¹²⁵I radioimmunotherapy, because of their more even distribution. It also suggests that a large number of binding sites per cell may be a disadvantage if more ¹²⁵I is bound than is necessary to kill the cell, because this may slow Ab penetration.

INTRODUCTION

In the search for the “perfect” Ab for RIT, emphasis has been placed on identifying Abs of high affinity, because they would be expected to bind tightly to the antigen-bearing tumor cells and thus enhance tumor cell killing. The suggestion has been made (1, 2) that a lower Ab affinity may allow enhanced penetration into tumors, thus producing a more even dose distribution. More detailed theoretical studies suggest that increasing Ab affinity would result in a more heterogeneous Ab distribution. More detailed theoretical studies suggest that increasing Ab affinity would result in a more heterogeneous Ab distribution. In larger tumors, lower affinity Abs would produce a more homogeneous dose distribution (4).

Affinity may be particularly important with RIT using ¹²⁵I, because of its very short range. ¹²⁵I decays by electron capture and internal conversion, followed by the production of Auger electrons. The electrons, γ-rays, and X-rays produced have very low energy (<40 keV) and, thus, only a subcellular range of cytotoxicity at the ¹²⁵I concentrations that are achievable in RIT. ¹²⁵I deposits most of its energy within a few angstroms, with a pattern of toxicity like that of other high linear energy transfer emitters, such as α-particles. However, the energy deposited decreases by a factor of 10 at a distance of 17 Å, and at 40 Å the energy is markedly reduced and has a low linear energy transfer-like effect (5, 6). It thus becomes clear that, for ¹²⁵I RIT, a cell must be targeted and the radiolabeled Ab must be internalized in order for the cell to be killed. It is, therefore, essential to have a uniform distribution of radiolabeled Ab. This is in contrast to β-emitters used for RIT, such as ¹³¹I, ¹⁸⁶Re, and ⁹⁰Y, which have a greater range and can kill cells without actually targeting them.

The purpose of this study was to examine the role of Ab affinity in the efficacy of ¹²⁵I RIT. Two Abs, 17-1A and 323/A3, have differing affinities but both bind to an antigen found in many gastrointestinal and other cancers. Both Abs are internalized after binding. Their penetration and toxicity were studied in human colon cancer multicell spheroids, where it is easy to compare relative amounts of Ab penetration by autoradiography.

MATERIALS AND METHODS

Cell Line. LS174T, a human colon adenocarcinoma cell line (7), was used for these studies. It was grown as a monolayer in DMEM (GIBCO, Grand Island, NY), with 1.5 g/liter NaHCO₃, 10% fetal calf serum (GIBCO), and 2 mm L-glutamine, and was kept at 37°C in a humidified 5% CO₂/air incubator. Antibiotics were not added to the medium. Multicell spheroids were grown by plating cells over 2% Bactoagar (Difco, Detroit, MI). When the spheroids were approximately 80 μm in diameter, they were transferred to 500-ml spinner flasks containing 300 ml of complete medium, gassed with 5% CO₂/air, and kept at 37°C. Medium was changed three times per week. Spheroid diameter (D) was determined by measuring two perpendicular diameters (A and B), using an inverted microscope; D was the geometric mean of A and B [D = (A x B)¹/²]. Spheroid volume (V) was calculated from the equation V = 4π(D/2)³/³.

Monoclonal Antibodies. Two murine monoclonal antibodies which bind to the same antigen were used for these studies. 17-1A, an IgG₂a from an immunization with a human colon cancer cell line, binds to a M, 30,000–40,000 glycoprotein (8, 9) and has been used extensively both experimentally (10–14) and in clinical trials (15). 323/A3, an IgG₁, was obtained from an immunization with a human breast cancer cell line and has been shown to bind to a M, 43,000 glycoprotein on the cell membrane (16). Pak et al. (17) have compared these two Abs by using the HT29 human colon cancer and MCF-7 human breast cancer cell lines and determined from competition studies that they recognize a similar antigen. They reported affinities of 1.7–3.4 x 10⁸ and 5.2 x 10⁸ M⁻¹ for 323/A3 and 17-1A, respectively. An affinity of 7 x 10⁸ M⁻¹ has also been reported for 17-1A (18). Although these two Abs are not of the same isotype, this should not be a problem in these in vitro studies. However, in vivo studies would not be comparable, because of...
the possible differences in Fc interactions with effector cells. R11D10, an IgG2a which binds to cardiac myosin, was used as an irrelevant control Ab (19).

**125I-Ab Labeling.** Antibodies were labeled using a modification of the chloramine-T method, with 11–12 mCi 125I/mg Ab (20, 21). Labeled Ab was purified over a PD-10 column (Pharmacia Inc., Piscataway, NJ). The percentage of activity bound to Ab was determined by trichloroacetic acid precipitation and was always >95%. Ab specific activity ranged from 10 to 11 mCi/mg.

**Immunoreactivity.** Immunoreactivity of labeled Ab was determined using a live cell assay, under conditions of antigen excess. Late logarithmic phase LS174T cells were resuspended at 5 x 10^6 cells/ml, and serial dilutions (1:2) were made. Half of the samples were preincubated for 30 min at 4°C with excess (333 nM) unlabeled Ab. The cells were then mixed 1:1 with 125I-Ab, at a final Ab concentration of 0.133 nM, and were incubated at 4°C for 2 h. Aliquots were then spun through oil [1:2 dibutyl:bis(2-ethylhexyl) phthalate oils], as described previously, to separate the cells from the medium (22). The cell pellet and the supernatant were then counted in a well-type gamma-counter (Packard, Downers Grove, IL). The immunoreactive fraction was determined using the technique of Lindmo et al. (23). Cell concentrations from 5 x 10^6 to 1.6 x 10^7 cells/ml were used with 125I-323/A3 and 125I-17-1A concentrations of 0.167 and 0.033 nM, respectively. The procedure was the same as described above. The immunoreactive fraction is the y-intercept of a linear regression of a plot of the inverse of the cell concentration versus the inverse of the fractional Ab binding.

**Antibody Affinity Determination.** LS174T cells were used at a concentration of 5 x 10^6 cells/ml and were mixed 1:1 with Ab. A constant concentration of 125I-Ab was used (1.67 nM final concentration), combined with increasing concentrations of unlabeled Ab. The cell/Ab mixture was incubated at 4°C for 2 h, and aliquots were spun through oil as described above. The results were analyzed using a computer program for Scatchard analysis (LIGAND; National Institutes of Health, Bethesda, MD). Affinities were also determined by correcting for immunoreactive fraction, using the method of Badger et al. (24).
Spheroid Antibody Uptake Studies. LS174T spheroids of approximately 1-mm diameter were rinsed twice in DMEM containing 1% fetal calf serum and were distributed at 6 spheroids/3-ml tube. $^{125}$I-Ab in 1% DMEM at 4°C was added to each tube, at a concentration of either 10 or 100 nM. Triplicate samples were incubated with mixing at 4°C for 1, 6, or 24 h. Incubation at 4°C prevents spheroid growth, thus avoiding possible errors in determining the depth of Ab penetration. The spheroids were then spun through oil as described above, counted in a gamma-counter, and flash-frozen for autoradiography (see below). One set of samples was incubated for 4 h, rinsed in 1% DMEM, counted in a gamma-counter, and resuspended in 1% DMEM for another 24 h, in order to evaluate Ab wash-out.

Monolayer and Spheroid Toxicity Studies. Attached monolayer cultures were incubated for 48 h in $^{125}$I-Ab, at an Ab concentration of 27 nM and an $^{125}$I concentration of 43 µCi/ml. The cells were then harvested, washed, counted in a gamma-counter, and plated for clonogenic assay. For the spheroid toxicity studies, 11-day-old LS174T spheroids were passed through nylon mesh screens, to obtain spheroids between 500 and 700 µm in diameter, and approximately 200–300 spheroids were aliquoted per spinner flask (total volume, 300 ml). Either $^{125}$I-17-1A, $^{125}$I-323/A3, $^{125}$I-R11D10, or unlabeled 17-1A was added to each flask, at an Ab concentration of 20 nM and an $^{125}$I concentration of 31–37 µCi/ml. Untreated spheroids were used as controls. The flasks were then gassed with 5% CO$_2$/air and placed in a 37°C water bath behind appropriate lead shielding. On days 3, 5, and 7, spheroid samples were removed, washed four times in 1% DMEM, and counted in a gamma-counter, diameters were measured, and the spheroids were dissociated and plated for clonogenic assay. Dissociated cells were plated to yield 25–100 colonies/plate, incubated for 18 days, and stained with crystal violet, and colonies containing >50 cells were scored. A feeder layer of 10$^5$ lethally irradiated cells/plate was used. Three separate experiments were performed.

Autoradiography. Spheroids were flash-frozen in optimal cutting temperature (OCT) embedding compound (Miles Inc., Elkhart, IN) and sectioned at 10-µm thickness. The slides were then placed in phosphate-buffered saline to remove the embedding compound, fixed in acetone, and dipped in NTB3 emulsion (Kodak, Rochester, NY). After appropriate time intervals at 4°C, the slides were developed using D19.
RESULTS

Immunoreactivity. Fig. 1 shows the fraction of $^{125}$I activity that was cell bound after 2 h of incubation at 4°C. Maximum binding of $^{125}$I-323/A3 was 62%, and this was significantly inhibited (98-99%) by preincubation with unlabeled 323/A3. Preincubation with unlabeled 17-1A had no effect on binding of $^{125}$I-323/A3. Maximum binding of $^{125}$I-17-1A was <10%, and inhibition by unlabeled 323/A3 was much more than by 17-1A itself. Immunoreactive fractions were 0.75 for 323/A3 and 0.30 for 17-1A.

Antibody Affinity Determination. Scatchard plots for the two Abs are shown in Fig. 2. The affinities ($K_a$) of 17-1A and 323/A3 were determined to be $5.15 \times 10^7$ and $1.87 \times 10^9$ M$^{-1}$, respectively. When corrections were made for immunoreactive fraction, the affinities were reduced by factors of 0.44 and 0.34, respectively. Displacement studies, using $^{125}$I-17-1A or $^{125}$I-323/A3 with increasing amounts of unlabeled 323/A3 or 17-1A, respectively, were also done. Even with a 105-fold excess of 17-1A, it was possible to inhibit $^{125}$I-323/A3 binding by only 20%. On the other hand, a 2-fold excess of 323/A3 was able to inhibit $^{125}$I-17-1A binding by 50%, and an 8-fold excess virtually eliminated $^{125}$I-17-1A binding. The number of binding sites/cell was estimated from these data to be $1-2 \times 10^6$ for both Abs.

Spheroid Antibody Uptake Studies. Ab binding in spheroids at 4°C, expressed as fmol Ab bound/mm$^3$ spheroid volume, is shown in Fig. 3. Binding continued to increase throughout the 24-h incubation period, and binding of the two Abs was approximately equivalent. A 10-fold increase in Ab concentration produced a 4.7-fold increase in binding for $^{125}$I-17-1A and a 2.6-fold increase for $^{125}$I-323/A3. A 4-h incubation in $^{125}$I-Ab followed by 20 h in medium alone produced 28% Ab wash-out with $^{125}$I-17-1A and 2.4% with $^{125}$I-323/A3, as might be expected, given the difference in their affinities.

Monolayer and Spheroid Toxicity Studies. In the monolayer toxicity studies, the binding of $^{125}$I-323/A3 was 16 times that of $^{125}$I-17-1A after 48 h at 37°C ($837$ versus $53$ fmol/10$^5$ cells). The surviving fractions for cells treated with $^{125}$I-323/A3 and $^{125}$I-17-1A were $6.4 \times 10^{-4}$ and 0.29, respectively, a difference of 450-fold. In the spheroid experiments, the spheroids in all groups continued to grow, although the treated spheroids grew slightly more slowly (Fig. 4). As in the monolayers incubated at 37°C, the binding of $^{125}$I-323/A3 was much more than that of $^{125}$I-17-1A (up to 8-10 times) (Fig. 4). However, despite this difference, $^{125}$I-323/A3 was only slightly more toxic than $^{125}$I-17-1A (Fig. 5). Unlabeled 17-1A and $^{125}$I-R11D10 (nonspecific Ab) produced minimal toxicity. When the data were corrected for differences in binding, by plotting survival against cumulative activity bound, expressed as $\mu$Ci-h/mm$^3$ spheroid volume, $^{125}$I-17-1A was found to be much more toxic than $^{125}$I-323/A3 (Fig. 5). The slopes of the two curves are significantly different ($P < 0.0001$).

The rate of $^{125}$I-Ab fall-off from the cells plated for clonogenic assay was determined, because this would influence the total radiation dose to the cells. Approximately 40% of the counts were lost from the cells during spheroid dissociation. Forty to 60% of the remaining counts were lost during subsequent resuspension and plating of cells. By 24 h after plating, only about 15% of the original activity was still on the cells. The activity was lost slightly more rapidly by 17-1A (12% activity remaining versus 18% for 323/A3).

Autoradiography. As reported previously (25, 26) and confirmed by these studies, penetration of whole Ab into multicell spheroids is relatively slow. At 4°C, there was little $^{125}$I-Ab penetration into the spheroids for either Ab after 1 or 6 h. After
24 h, there was approximately 100-μm penetration by the lower affinity 17-1A but still very little penetration by 323/A3 (Fig. 6). Contact film autoradiographs did not show significantly different results from liquid emulsion. At 37°C, autoradiographs at 3, 5, and 7 days showed much enhanced penetration of 17-1A, compared to 323/A3 (Figs. 7 and 8). At 37°C there is some spheroid growth, so some of the apparent penetration could be due to incorporation of bound Ab into the depths of the spheroids as new cells appear on the surface. However, the autoradiographs clearly showed that this factor is of minor importance, because radionuclide reached the center of the spheroids treated with 17-1A and not those treated with 323/A3. The spheroids grew similar amounts with each treatment.

DISCUSSION

These studies were intended to determine the importance of Ab affinity in the efficacy of 125I-RIT. 323/A3 was found to have an affinity 36 times that of 17-1A. From these studies, it appears that these two Abs bind to different epitopes of the same antigen. The binding site for 17-1A is probably situated such that, when the binding site for 323/A3 is occupied, 17-1A cannot reach its binding site, inasmuch as preincubation with 323/A3 completely blocks binding of 125I-17-1A. On the other hand, preincubation with 17-1A has no effect on 323/A3 binding. These observations cannot be explained by differences in affinity alone. Pak et al. (17) have also shown that binding of 125I-17-1A is more easily inhibited by unlabeled 323/A3 than is the binding of 125I-323/A3. It should be noted that, in monolayer cells at 4°C under conditions of Ab excess, binding of both Abs was similar (data not shown). Because maximal binding should be determined by the number of antigenic sites under these conditions, this is further evidence that the Abs bind to the same antigenic site.

Another interesting observation is the difference in binding of 125I-17-1A in spheroids at 4°C and 37°C. The binding of 17-1A is similar to that of 323/A3 at 4°C but is 8- to 10-fold less at 37°C. Given the findings in monolayer cells, which suggested that the 17-1A binding site is less accessible than the 323/A3 binding site, it may be that changes in membrane fluidity at 37°C lead to a physical barrier to Ab access to the
17-1A binding site. This could be due to a glycosylation site near the 17-1A binding site, which is known to be associated with reduction in Ab affinity. It also could be that, after internalization (which does not happen at 4°C), 17-1A is handled differently, resulting in down-regulation of receptor recycling, whereas 323/A3 receptors may be recycled more efficiently.

The most important finding in this study is that, despite the much lower binding of $^{125}$I-17-1A, it was nearly as toxic as $^{125}$I-323/A3 to spheroids. When corrections were made for the reduced binding, $^{125}$I-17-1A was found to be much more efficacious than $^{125}$I-323/A3. One possible explanation could be that $^{125}$I-17-1A is internalized differently and brought closer to the DNA than is $^{125}$I-323/A3, resulting in a high linear energy transfer pattern of cell killing. However, the toxicity studies in monolayers do not support this, because the reduced binding of $^{125}$I-17-1A in this situation was associated with significantly less killing than with $^{125}$I-323/A3. Therefore, it must be the more uniform distribution of $^{125}$I in the spheroids, due to the lower affinity of 17-1A, that led to the enhanced killing by $^{125}$I-17-1A. In fact, it has been shown in another laboratory that the affinity of 17-1A is lower when measured at 37°C than at 4°C, which further supports our conclusions on the importance of affinity on Ab penetration.

Because of the short range of $^{125}$I, a cell must be targeted directly by $^{125}$I-Ab in order to be killed efficiently. There was probably more binding of $^{125}$I-323/A3 in the superficial layers of the spheroids than was necessary for cell killing, and there was very little binding in the deeper cell layers, resulting in inadequate toxicity in this region. On the other hand, $^{125}$I-17-1A was much more evenly distributed, resulting in improved cell killing in the deeper layers without "overkill" in the superficial layers.

From these studies, it can be concluded that in $^{125}$I RIT, for equivalent binding, an Ab of lower affinity may be more efficacious than one of higher affinity. These results also suggest that a lower number of antigenic binding sites per cell may be advantageous with high affinity Abs, because this would allow more rapid penetration of radiolabeled Ab into the depths of the tumor, in order to overcome the "binding site barrier" (3). There is likely an optimal Ab affinity for each tumor, which is determined in part by the number of antigenic binding sites per 4 H. Takahashi, Massachusetts General Hospital, personal communication.
cell. Before these results can be applied clinically, it is also important to determine the therapeutic ratio (tumor/normal tissue binding ratio) over the treatment time, because this ratio will ultimately determine the maximum dose of radiolabeled Ab that can be administered. A detailed understanding of these factors should help in optimizing the tumor dose in 125I RIT.

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