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Tumor Pretargeting: Role of Avidin/Streptavidin on Monoclonal Antibody Internalization

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Radioimmunodetection of tumor can be improved by introducing a two-step system in which radiolabeled streptavidin is administrated after the injection of a biotinylated monoclonal antibody (MAb) (two-step) or radiolabeled biotin is injected after biotinylated MAb and avidin (three-step). The anti-carcinoembryonic antigen (CEA) MAb FO23C5 has been recently exploited in a three-step protocol based on the avidin–biotin system. The anti-folate receptor (FR) MAb MOv18 has proven suitable for radioimmunodetection of ovarian cancer using directly radiolabeled MAb or in a two-step method. In this study, we analyzed the suitability of MOv18 in a three-step protocol in ovarian carcinoma patients and the internalization events after formation of the MOv18–avidin complex. **Methods:** Selected patients with documented metastatic lesions were enrolled in a three-step radioimaging analysis with biotinylated MOv18 and FO23C5, avidin and ^{111}In-labeled biotin. Two-step internalization experiments were conducted in vitro with MOv18 and MOv19 MAbs on the FR-overexpressing IGROV1 cell line and with the anti-CEA MAb FO23C5 on the LS174T cell line. Cells were incubated sequentially with biotinylated MAb and ^{125}I-labeled streptavidin or with ^{125}I-biotinylated MAb and cold streptavidin. **Results:** In the in vivo study, SPECT revealed the majority of metastatic lesions in patients injected with biotinylated MOv18; however, the tumor-to-background ratio was relatively low. In the in vitro study, a consistent internalization was induced by antigen-biotinylated MAb–streptavidin complex formation at the cell surface in both antigenic systems analyzed. However, the extent of internalization was lower in the CEA model. **Conclusion:** The internalization ability of avidin suggests its potential clinical application for delivering toxic agents in a two-step approach (biotinylated MAb + avidin conjugate). The suitability of a given MAb for three-step clinical applications (biotinylated MAb + avidin + biotin) should be previously investigated by using appropriate in vitro experiments.

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The avidin/streptavidin–biotin complex has become an extremely useful and versatile detection intermediate in a variety of biological and analytical systems (*1–3*). Its high binding affinity (10^{-15} *M*) ensures binding at extremely low reagent concentrations and binding stability even under extreme conditions (*4*). Recently, the use of this system has been extended to include in vivo procedures such as radioimmunodetection (RID) (*5–8*). Radiolocalization studies have shown that target-to-nontarget radioactivity ratios and radioimaging analyses can be significantly improved by introducing a two-step or three-step system in which radiolabeled avidin or streptavidin is administered after the injection of a biotinylated ligand or radiolabeled biotin is injected after a streptavidin-conjugated antibody or an avidin chase of unbound ligand (*9–12*). The advantages and limitations of the two methods are described elsewhere (*13,14*). The avidin–biotin system, applied to RID, allows the use of short-half-life radionuclides and has been shown to enhance the applicability and effectiveness of radio-immuno-guided surgery (*10*). However, in this system, the three-step approach is feasible only when the ligand–avidin complex is still present on the membrane at the time that radiolabeled biotin is administered.

The major area of clinical application of the avidin–biotin complex is the detection of tumor markers bound by monoclonal antibodies (MAbs). Several markers of human carcinomas have been identified so far, and some of them have been exploited in RID and radioimmunotherapy, based on the tumor-restricted and homogeneous overexpression of these markers (*15,16*). The folate receptor (FR), which is recognized by MAbs

TABLE 1
Summary of Radioimmunolocalization Data

MAb	Method	No. of cases	T-to-BKG ratio (range)	Ref.
MOv18	Two-step	15	9–30	Paganelli et al. *(21)*
	Three-step	4	1.7–3	This study
FO23C5	Three-step	108	5.5–12	Paganelli et al. *(7)* Paganelli et al. *(28)*
	Three-step	7	8–10	This study

MOv18 and MOv19 (*17,18*), has proven suitable for RID and radioimmunotherapy using directly radiolabeled MAb (*19,20*). The use of MOv18 in a two-step method using biotinylated MAb followed by radiolabeled streptavidin gave excellent results (*21*) because the MAb is not readily internalized (*22*).

Preliminary data in a three-step protocol with MAb MOv18 gave rise, in ovarian carcinoma patients, to weaker imaging compared to the results with the two-step method. Although differences in pharmacokinetic must be considered with different antibodies, we addressed the question of whether this difference was also due, in our three-step method, to induction of internalization events after formation of the MAb–avidin complex. Here, we describe in vitro internalization studies with the two anti-FR MAbs, MOv18 and MOv19, and the anti-CEA MAb FO23C5 (*23*).

MATERIALS AND METHODS

Reagents

MAb MOv18 (IgG1) and FO23C5 (IgG1) were provided for clinical use; MAb MOv19 (IgG2a) was purified from culture supernatants by protein A affinity chromatography. The MAbs were biotinylated essentially as described (*7*) by Società Prodotti Antibiotici (Milan, Italy).

Pure hen egg avidin and streptavidin were obtained from Società Prodotti Antibiotici; ^{125}I-labeled streptavidin (specific activity, 40 μCi/μg) was purchased. Diethylenetriaminepentaacetic acid-conjugated biotin was purchased and radiolabeled as described (*7*).

Patients

Eleven patients with documented metastatic lesions of ovarian (FR-overexpressing; four patients) or CEA-overexpressing cancer (seven patients) were enrolled in the study after informed written consent was obtained.

The three-step protocol was performed essentially as described (*7*) but with an optimized timing of injection. Briefly, tumor pretargeting was performed as follows: 2 mg of biotinylated MAb was injected intravenously (first step), followed 36 hr later by a double injection of 2 mg of avidin (chase) plus 5 mg streptavidin over 10 min (second step), followed 24 hr later by a third injection of ^{111}In-labeled biotin (third step). Images were acquired with a 40-cm circular field rotating gamma camera equipped with a high-energy collimator and by selecting two 15% energy windows centered over the 173 and 247 keV photopeaks of ^{111}In. Whole-body images in anterior and posterior views were collected by SPECT (64 × 64 pixel matrix; 64 projections over 360°) 2 hr after the ^{111}In-labeled biotin injection.

In Vitro Study

Experiments were conducted with the FR-expressing human ovary carcinoma cell line IGROV1, in the cases of MOv18 and MOv19, and with the CEA-expressing human colon carcinoma LS174T, in the case of FO23C5. Cells were maintained in vitro, and the assays were conducted essentially as described (*22*).

Evaluation of Iodine-125-Labeled Streptavidin Internalization. Live cells were incubated for 1 hr at 37°C with cold biotinylated MAb (0.2 n*M*, in the case of MOv18 and MOv19, and 7 n*M*, in the case of FO23C5) or with medium alone as control and, after removal of unbound antibody, incubated at 37°C with equimolar concentrations of ^{125}I-labeled streptavidin. At various time intervals, duplicated aliquots of 200,000 cells were harvested, washed and assessed for total cell-associated radioactivity in a γ counter. Cells were incubated for 20 min at room temperature with 1 ml of 50 m*M* glycine-HCl/100 m*M* NaCl, pH 2.8. This treatment, known to dissociate antigen–antibody complex, allowed a discrimination between externally bound and internalized antibody (*24*). After washing, cells were assessed for residual radioactivity, and the percentage of internalization was evaluated (residual/total). The

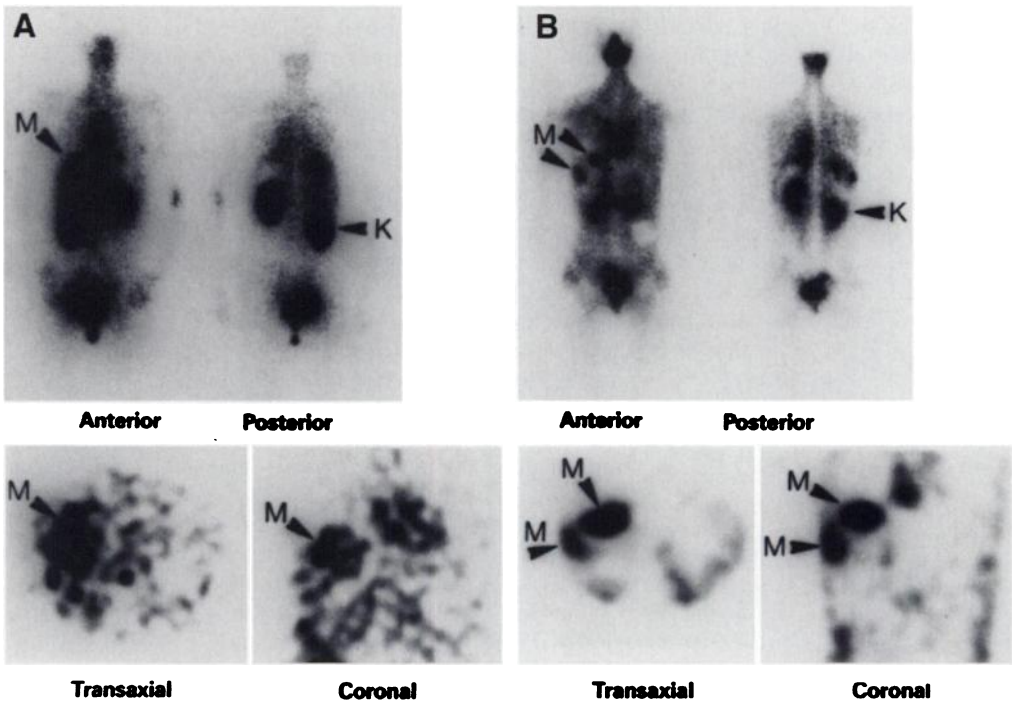

FIGURE 1. Three-step SPECT scan of carcinoma patients with liver metastasis injected with biotinylated MAb and recorded 2 hr after injection of ^{111}In-labeled biotin. Planar whole-body images are represented in anterior and posterior views. (A) Ovarian carcinoma patient injected with anti-FR MAb MOv18; (B) breast cancer patient studied according to the same protocol as in A but using anti-CEA MAb FO23C5 as the first step. K, kidneys; M, metastasis.

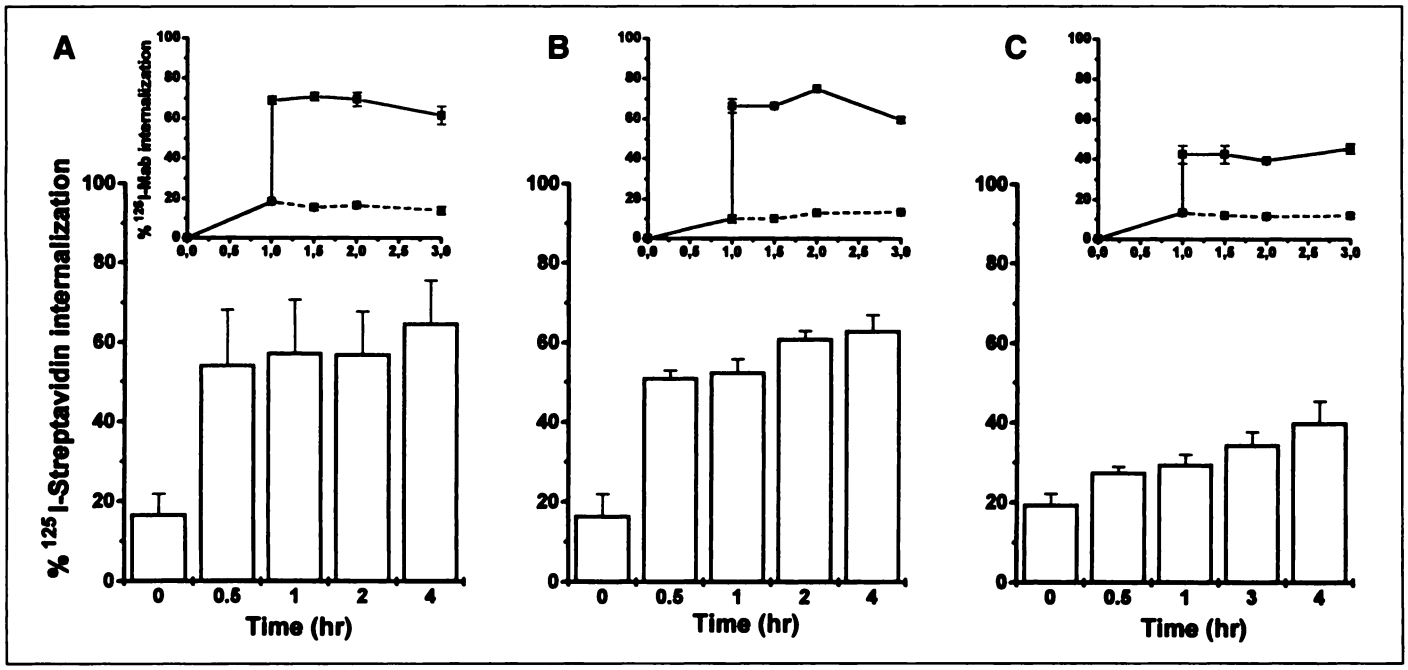

FIGURE 2. Time course of biotinylated MAb internalization. (A–C) Mean internalization of ^{125}I-labeled streptavidin–MAb complexes in 2–5 experiments; error bars (intra-assay variability) represent s.e. (Insets) Iodine-125-biotinylated MAb internalization; the percentage internalization was evaluated in the presence (solid line) or in the absence (dashed line) of cold streptavidin; error bars (inter-assay variability) represent s.d. (A) MAb MOv18 with IGROV1 cells; (B) MAb MOv19 with IGROV1 cells; (C) MAb FO23C5 with LS174T cells.

differences between measurements of each time interval were statistically evaluated by Student's t-test.

Evaluation of Iodine-125-Labeled MAb Internalization. Parallel experiments run under the conditions described above were conducted with directly ^{125}I-radiolabeled MAbs and cold streptavidin. The biotinylated MAbs were radiolabeled using the lactoperoxidase method (*22*) to a final specific activity of 8 μCi/μg. The differences between presence or absence of cold streptavidin and between each time interval were statistically evaluated by Student's t-test.

RESULTS

In Vivo Study

Due to its suitable pharmacokinetics (*19*), similar to those of isotype-matched MAbs, and to its excellent localization ability in a two-step method (Table 1), anti-FR MOv18 MAb seemed to be a good candidate for the three-step approach. Four ovarian carcinoma patients with known metastatic lesions were included in a three-step protocol imaging study. Although the lesions could be visualized in all of them, the resolution of imaging was lower [range of tumor (T)-to-normal tissue (BKG) ratios, 1.7–3] than that expected, based on the two-step protocol study (Table 1). Patients with similar metastatic lesions were studied with the same administration protocol using the anti-CEA FO23C5 MAb, which has been successfully applied in three-step RID (Table 1). The imaging acquired in seven patients and analysis by means of region of interest confirmed the good RID ability of FO23C5 (range of T-to-BKG ratios, 8–10).

An example of a SPECT scan of an ovarian carcinoma patient recorded after injection in the first step of MOv18 MAb is shown in Figure 1A. Indium-111-labeled biotin is rapidly excreted through the kidneys and is also pathologically accumulated (T-to-BKG ratio, 3) in the right lobe of the liver where metastatic ovarian carcinoma was documented by ultrasound. The hepatic lesions are better visualized in the tomographic SPECT study (transaxial and coronal views). Figure 1B shows the SPECT scan of a breast carcinoma patient with a CEA-overexpressing tumor recorded after injection in the first step of anti-CEA MAb FO23C5, as an example of good resolution by the three-step protocol. Note the higher uptake (T-to-BKG ratio, 8) in the liver metastatic lesions, both in planar and tomographic images.

In Vitro Study

To mimic the two-step protocol, cells were first incubated at 37°C with biotinylated MAbs and then with ^{125}I-labeled streptavidin to follow the internalization of the MAb–streptavidin complex. In the absence of biotinylated MAbs, the spontaneous internalization of ^{125}I-labeled streptavidin was negligible (less than 1%). In the case of anti-FR MAbs, the addition of ^{125}I-labeled streptavidin to cells pretargeted with biotinylated MAbs induced significant internalization of the MAb–streptavidin complex ($p < 0.05$). The uptake was nearly accomplished after 30 min, with around 60% internalized ^{125}I-labeled streptavidin (Fig. 2, A and B). In the case of biotinylated anti-CEA MAb, the addition of ^{125}I-labeled streptavidin induced a slow but constant increase of internalization that reached around 40% after 4 hr of incubation. Again, the uptake increment was significant ($p < 0.05$) only after the first time interval of observation (Fig. 2C). The proposed mechanism of internalization is a cell surface clustering of antigen–antibody complexes after ^{125}I-labeled streptavidin binding. To exclude the possibility that the intracellular radioactivity detected reflects cellular uptake of the ^{125}I-labeled streptavidin per se after clustering and not internalization of the complex, the internalization of ^{125}I-biotinylated MAbs after addition of cold streptavidin was analyzed (Fig. 2, insets). In the absence of streptavidin, all three MAbs induced less than 20% antigen–antibody complex internalization. After clustering of antigen–antibody complexes by addition of cold streptavidin, the percent of internalization was significantly increased ($p < 0.05$) to over 60% in the case of the two anti-FR MAbs (Fig. 2, A and B, insets) and around 40% with anti-CEA MAb (Fig. 2C, inset). The kinetics were very rapid even in the case of the anti-CEA MAb, and the small differences in uptake over the time were not significant. These

data confirmed the low intrinsic internalization ability of the analyzed MAbs.

DISCUSSION

Here we demonstrated that, when a two-step protocol was applied in vitro, a consistent internalization was induced by antigen-biotinylated MAb–streptavidin complex formation.

As a possible mechanism, we suggest that the homotetrameric structure of streptavidin causes a stable antigen clusterization, which occurs very rapidly due to the extremely high affinity of the streptavidin subunits for biotin (*3*). In those models in which antigen cross-linking is a crucial step in the cellular internalization, it will rapidly take place. However, this phenomenon takes place to different extents, depending on the antigenic system examined.

It is unlikely that the difference was due to the antigenic density because both FR and CEA were highly overexpressed in human tumors (*25,26*). Moreover, because the two non-cross-reactive anti-FR MAbs behave similarly, the intrinsic characteristic of the single epitope involved does not play a role.

A different topobiology of the FR and CEA molecules, i.e., cell surface clustering and membrane association with lipids and/or other cellular proteins (*26,27*), might account for the observed difference in increase in internalization induced by formation of the biotinylated MAb–streptavidin complex.

The higher internalization after MOv18- or MOv19-streptavidin complex formation, compared to that induced by FO23C5, correlated with the in vivo data, in which a lower resolution in imaging was observed. It is likely that, at least in our three-step method, the relatively low ^{111}In-labeled biotin uptake in ovarian cancer patients was due to induction of internalization events after the formation of the MOv18 Mab–avidin complex.

In the case of a rapid and massive internalization of the complex, the use of an alternative two-step method, i.e., streptavidin-conjugated MAb and radiolabeled biotin (*5*), could overcome the problem, allowing a great reduction in the waiting period before tracer injection.

CONCLUSION

We suggest that the suitability of a given MAb for three-step avidin–biotin clinical applications should be previously tested by an appropriate in vitro analysis of internalization. On the other hand, cellular internalization can also be an advantage. The antigen–antibody complex formation after avidin clustering suggests the clinical exploitation of avidin/streptavidin for delivering toxic agents, which must be internalized into the cells to exert their toxicity, in models in which internalization is not achieved by MAb alone. An avidin-toxic agent conjugate could be a universal reagent for tumor therapy.

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