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Influence of Cocktails of Labeled Monoclonal Antibodies on the Localization of Antibodies in Human Tumor Xenografts

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In order to evaluate the usefulness of cocktails of labeled monoclonal antibodies (MoAbs) recognizing different antigen molecules to localize human cancer xenografts, we have compared the potential of three MoAbs recognizing representative cancer-associated CA 19-9, 17-1A and CEA antigens when administered alone or in combination. Specific binding of radioiodinated $F(ab')_2$ fragments of these three MoAbs was observed to human colorectal cancer cell lines SW1116, LS180 and Co-3. The percentage of *in vitro* cell binding of a cocktail of any two MoAbs to cancer cells was equal to the average of those obtained with the two MoAbs alone. The three MoAbs were preferentially localized in tumor tissues xenografted in nude mice. When cocktails of any two MoAbs were used, the obtained tumor-to-normal tissue ratios and percent of injected dose per gram of tumor were between the levels obtained for each MoAb when administered alone, in all three tumors transplanted in nude mice. These data suggest that, although cocktails of labeled MoAbs recognizing different antigens may extend the spectrum of tumor specificities, their use does not improve the tumor localization ability of MoAb-conjugates.

Key words: Monoclonal antibody cocktail — CA 19-9 — 17-1A — Carcinoembryonic antigen — Tumor targeting

The development of the hybridoma technique has provided numerous murine MoAbs specific for malignant tumors, and many MoAbs[4] conjugated with radionuclides, toxins or chemotherapeutic agents have been employed for the diagnosis and/or therapy of human tumors.[1-7] The use of MoAbs depends on their accumulation and retention in tumor tissues. In contrast to the successful treatment of animal tumors using MoAb-conjugates, low tumor uptake and low tumor-to-normal tissue ratios limit the use of MoAb-conjugates for the therapy of human malignancies.[5-8] It is recognized that the antigenic expression on tumor tissues recognized by anti-tumor MoAbs is heterogenous among human tumors and even within a tumor.[9,10] Variations in the expression and density of antigens on individual tumor cells could explain difficulties in detecting all known lesions using a single labeled MoAb.[1,2,8,11,12] Use of a cocktail of MoAbs might be one approach to label a greater percentage of different cells and/or to increase the density of redioactivity in tumor tissues.[12]

The present studies were performed to determine if there are advantages to the use of labeled MoAbs as a mixture or cocktail. In this paper, we have compared the *in vitro* cell binding properties and *in vivo* tumor localization of $F(ab')_2$ fragments of three radiolabeled representative anti-tumor MoAbs, recognizing CA 19-9, 17-1A and CEA antigens, used alone or in combination. These three MoAbs have been widely employed for the serodiagnosis, immunoimaging or immunotherapy of patients with colorectal and pancreatic cancers.[3,8,13-15]

MATERIALS AND METHODS

Monoclonal antibodies $F(ab')_2$ fragments of 1116-NS-19-9 (IgG_1),[16,17] CO 17-1A (IgG_{2a})[13] and an anti-CEA MoAb designated as F33-104 (IgG_1),[15,18] as well as normal murine immunoglobulin controls were used in the present study. $F(ab')_2$ fragments of 1116-NS-19-9 and CO 17-1A were provided by Centocor (Malvern, PA), and $F(ab')_2$ fragments of normal murine immunoglobulin were purchased from Organon Teknika Corp. (West Chester, PA). MoAb 1116-NS-19-9 recognizes a sialylated fucopentose II carbohydrate determinant, designated CA 19-9, expressed on circulating glycoprotein antigens[19] and anti-CEA MoAb (F33-104) is reactive with the CEA-specific protein part of CEA.[15]

Radiolabeling The Chloramine-T method was employed for the radioiodination of MoAbs without loss of antigen-binding activities, as described previously.[20] Specific

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[4] The abbreviations used are: MoAb, monoclonal antibody; Ab, antibody; CEA, carcinoembryonic antigen; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

activities of radiolabeled MoAbs were between 4 and 7 mCi/mg for ^{125}I-labeled antibodies and were adjusted to 3 mCi/mg by adding corresponding unlabeled MoAbs. Immunoreactive fractions of ^{125}I-labeled F(ab′)$_2$ fragments of MoAb 19-9, 17-1A and F33-104 were calculated as about 0.60, 0.60 and 0.40, respectively, according to the method of Lindmo *et al.*[21]

Three cocktails were prepared by mixing two ^{125}I-labeled MoAbs with similar radioactivity, that is combinations of 19-9 plus 17-1A, 19-9 plus anti-CEA and 17-1A plus anti-CEA MoAbs, and contained total radioactivity of 3 mCi/mg Ab.

***In vitro* binding studies** Human colorectal cancer cell lines SW1116, LS180 and Co-3 (obtained by courtesy of the Experimental Animal Center Research Foundation, Kawasaki) were xenografted in nude mice and maintained by serial subcutaneous implantation.[10, 22, 23] Tumor cell suspensions were prepared from xenografts of SW1116, LS180 and Co-3 in nude mice by passing tumor specimens through a stainless steel mesh.[10] Red blood cells were removed by hemolysis. Tumor cells (1×10^6) were suspended in 100 μl of PBS buffer.

In order to obtain equal amounts of total radioactivity and antibody dose, ^{125}I-labeled MoAbs alone were diluted to two concentrations; 30 nCi (about 34,000 cpm)/10 ng Ab or 15 nCi/5 ng Ab in 100 μl PBS containing 0.25% w/v PBS/BSA. Two ^{125}I-labeled MoAbs were mixed together, and adjusted to 30 nCi/10 ng in 100 μl of PBS/BSA. One hundred microliters of ^{125}I-labeled MoAb alone (30 nCi/10 ng or 15 nCi/5 ng) or an equal mixture of two MoAbs (30 nCi/10 ng) was incubated with 1×10^6 tumor cells in 100 μl of PBS in 5.7× 46 mm microcentrifuge tubes for 2 h at 4°C. After centrifugation, the supernatant was aspirated and the pellet was cut out. The percentage of radioactivity bound specifically to cells was determined by subtracting the non-specific binding of ^{125}I-labeled F(ab′)$_2$ fragment of normal murine immunoglobulin.

***In vivo* biodistribution** Nude mice bearing human colorectal cancer xenografts of SW1116, LS180 or Co-3 were given iv injection of ^{125}I-labeled MoAbs alone or a combination of two MoAbs (1 μCi/20 μg). The administered antibody dose was adjusted to 20 μg per mouse by mixing ^{125}I-labeled and unlabeled corresponding MoAb. The injected MoAbs cocktails contained an equal mixture of the two MoAbs (each 0.5 μCi of ^{125}I radioactivity and 10 μg of antibody). Tumors weighing 0.5 to 1.0 g were used in the present study. Thyroids were blocked with drinking water containing 0.1% w/v potassium iodide during the study. Animals were killed 48 h after the administration and the organs were weighed and counted. The biodistribution data were represented as a percentage of the injected dose per gram normalized to a 20 g mouse and as tumor-to-normal tissue ratios, and also evaluated by the use of a localization index, calculated as the ratio of tumor-to-blood ratio of specific antibody to that of control immunoglobulin.[24]

Imaging study Animal images were made using a gamma camera (Searl, Chicago, IL) equipped with a pinhole collimator following iv administration of 100 μCi of ^{131}I-labeled F(ab′)$_2$ fragments of 19-9, 17-1A and anti-CEA MoAbs. Images were taken at 6, 24 and 48 h after the injection.

RESULTS

***In vitro* cell binding** Colorectal cancer cell lines SW1116, LS180 and Co-3 express CA19-9, 17-1A and CEA antigens on their cell surface membranes, and radioiodinated 19-9, 17-1A and anti-CEA MoAbs showed specific bindings to these tumor cells. As shown in Fig. 1, 47.8, 22.2 and 16.0% of added ^{125}I-labeled F(ab′)$_2$ fragments of 19-9, 17-1A and anti-CEA MoAbs bound to 1×10^6 SW1116 tumor cells, respectively. The bound percentage of radioactivity of 30 nCi/10 ng MoAb was similar to that of 15 nCi/5 ng MoAb in any combination of MoAbs and tumor cells, indicating that these concentrations of radiolabeled MoAbs were non-saturating. When two ^{125}I-labeled MoAbs were used in combination, the percent binding to SW1116 cells was close to the mean of those of each MoAb when used alone, that is, 33.1, 32.7 and 18.7% for coktails of 19-9 plus 17-1A, 19-9 plus anti-CEA, and 17-1A plus anti-CEA, respectively. Similar results were observed in cell-binding assays using equal mixtures of any two of 19-9, 17-1A and anti-CEA MoAbs and LS180 or Co-3 cells (Fig. 1).

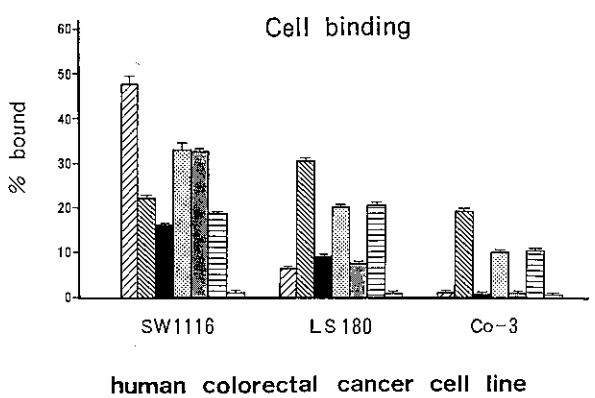
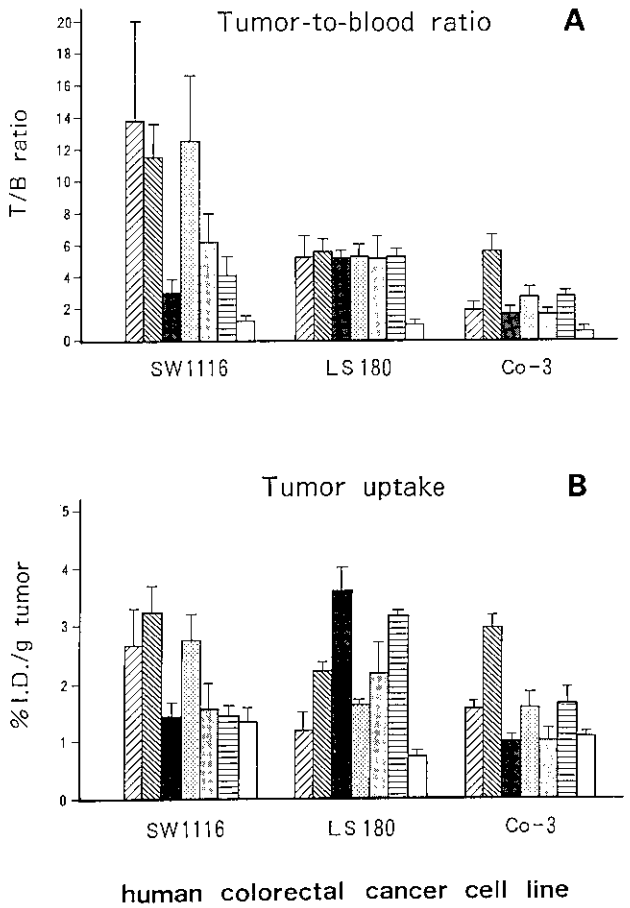


Fig. 1. Specific binding of ^{125}I-labeled F(ab′)$_2$ fragments of MoAbs alone or in combination at the antibody dose of 10 ng to human colorectal cancer cell lines SW1116, LS180 and Co-3. Columns and vertical bars represent the mean and SD of these experiments. ▨ 19-9, ▧ 17-1A, ■ CEA, ▩ 19-9+17-1A, ▩ 19-9+CEA, ▤ 17-1A+CEA, □ control.



Tumor uptake of labeled antibodies The ^{125}I-labeled $F(ab')_2$ fragments of 19-9, 17-1A and anti-CEA MoAbs were localized preferentially in three colorectal cancer cell lines, SW1116, LS180 and Co-3, xenografted in nude mice, as shown in Fig. 2A and B. Tumor-to-normal tissue ratios of radioactivity increased with time, and scintigrams clearly visualized the transplanted tumor at 48 h after injection as shown in Fig. 3. Tumor-to-blood ratios of SW1116-bearing nude mice obtained at 48 h after injection of 19-9, 17-1A and anti-CEA MoAbs alone were 13.8, 11.5 and 3.2, respectively, while that of control antibody was 1.4. In comparison with each MoAb alone, the combined use of two MoAbs did not increase the localization of radioactivity in the tumor, but the tumor uptake was equivalent to the average of those of the two MoAbs individually. Tumor-to-blood ratios obtained at 48 h after injection of equal mixtures of 19-9 plus 17-1A, 19-9 plus anti-CEA, and 17-1A plus anti-CEA MoAbs in SW1116-bearing nude mice were 12.5, 6.0 and 4.2, respectively. Similar results were observed in nude mice bearing LS180 and Co-3 tumors, in

Fig. 2. Tumor-to-blood ratios (A) and percent of injected dose per gram of tumor (B) for xenografts of human colorectal cancer cell lines SW1116, LS180 and Co-3 in nude mice 48 h after injection of 20 μg of ^{125}I-labeled $F(ab')_2$ fragments of MoAbs alone or in combination. Columns and vertical bars represent the mean and SD of 4 to 10 nude mice. ▨ 19-9, ▧ 17-1A, ■ CEA, ▩ 19-9+17-1A, ▩ 19-9+CEA, ▤ 17-1A+CEA, □ control.

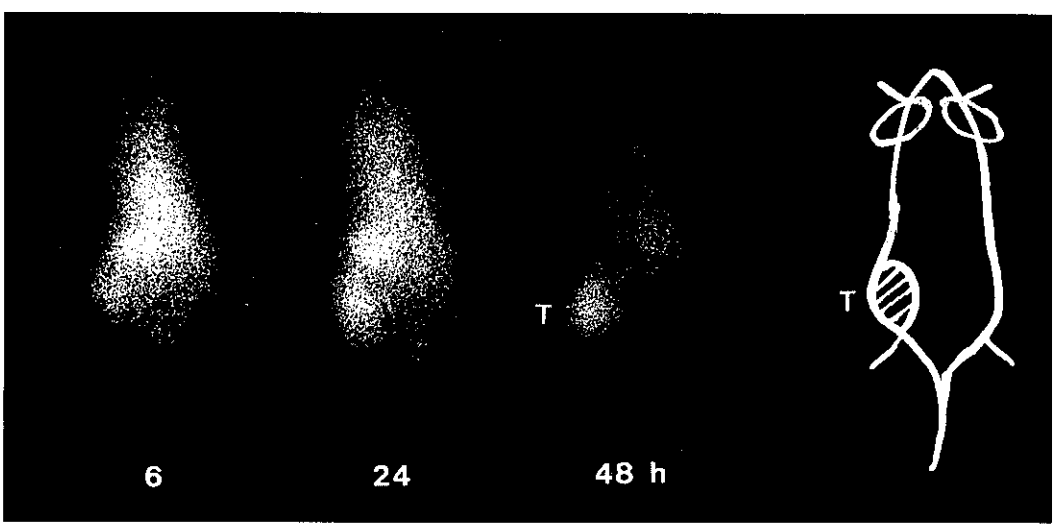


Fig. 3. Scintigrams of SW1116 tumor-bearing nude mouse injected with 100 μCi of ^{131}I-labeled $F(ab')_2$ fragments of F33-104 MoAb recognizing CEA at the antibody dose of 30 μg. Scans were taken at 6, 24 and 48 h after injection. T: tumor.

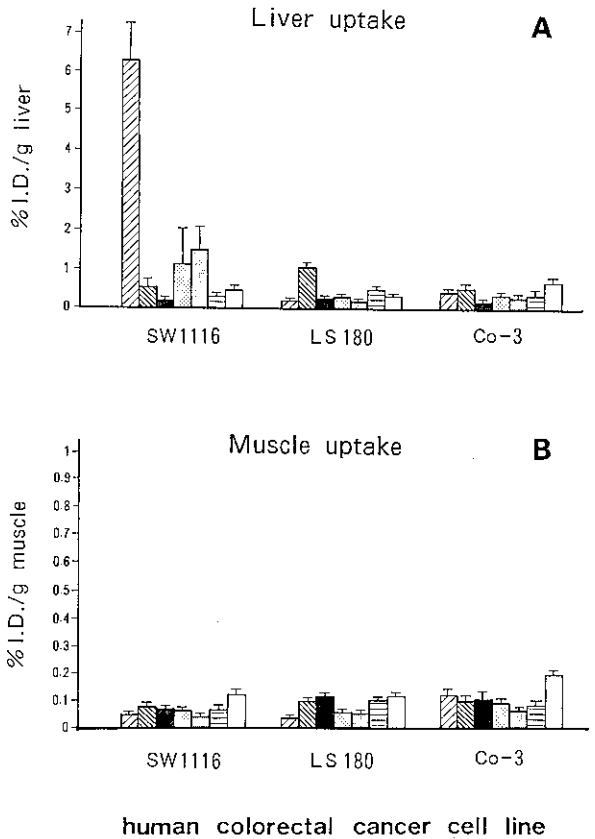


Fig. 4. The distribution of ^{125}I-labeled F(ab')$_2$ fragments of MoAbs alone or in combination at the dose of 20 μg in liver (A) and muscle (B) in nude mice bearing SW1116, LS180 and Co-3 tumors 48 h after injection. Columns and vertical bars represent the mean and SD of 4 to 10 nude mice. ▨ 19-9, ▧ 17-1A, ■ CEA, ▩ 19-9+17-1A, ▩ 19-9+CEA, ▤ 17-1A+CEA, □ control.

that a mixture of MoAbs did not offer a higher uptake in the tumor than the average of the uptakes of the single MoAbs. As shown in Fig. 4, the distributions of these MoAbs in normal tissues were lower than that in tumor except for high liver deposition with 19-9 antibody, and the mixed use of two MoAbs resulted in an averaged localization. An equal mixture of any two MoAbs did not improve the tumor-to-normal tissue ratio of radioactivity obtained with the better MoAb alone in any of these three tumor models.

DISCUSSION

Many anti-tumor MoAbs have been employed to carry radionuclides, toxins or anti-neoplastic drugs to tumor tissues, which are generally considered to be composed of heterogeneous populations of cells differing in antigen expression.[9, 10] Mixed use of MoAbs recognizing different antigens expressed within a tumor cell population or within the same tumor cell would be expected to be more effective for tumor localization than when only one antigen is targeted.[6, 9, 12, 25]

In order to compare the tumor localization ability of single MoAbs with that of combinations of two MoAbs recognizing separate antigen molecules, we have used three anti-tumor MoAbs, which detect representative gastrointestinal cancer-associated antigens CA 19-9, 17-1A and CEA, and three human colorectal cancer cell lines, SW1116, LS180 and Co-3. These cancer cell lines express CA 19-9, 17-1A and CEA antigens on their surface membranes and were successfully xenografted in nude mice. The ^{125}I-labeled F(ab')$_2$ fragments were localized preferentially in these xenografted colorectal cancer tissues, when administered alone or in combination. The tumor-to-blood ratio of each MoAb was significantly higher than that of control antibody, resulting in localization index values of 9.9 for 19-9, 8.2 for 17-1A and 2.3 for anti-CEA MoAbs in SW1116 tumors at 48 h after injection. However, liver retention of 19-9 antibody was high in SW1116-bearing nude mice, since CA 19-9 with a molecular weight of more than 5,000,000 is released into the circulation and forms antigen-antibody complex, as previously described.[23]

In patients with malignant melanoma or colorectal cancer, the detection rate of tumor increased as the administered Ab dose was increased, and 20 mg or 40 mg of Ab has been clinically used for tumor localization.[1, 2] In our present study, the antibody dose infused was adjusted to 20 μg/mouse, which is within the range of clinical use,[1, 2, 7, 11] and F(ab')$_2$ fragments were employed to avoid nonspecific binding caused by Fc-receptor.[23] The tumor-to-normal tissue ratio increased with time and the dissection time of 48 h after injection was selected, since it matches well with the optimal time for clinical radioimmunoimaging,[1, 2, 11, 26] and the biodistribution data are directly comparable with those reported in other papers.[23, 27, 28]

In our present study, equal mixtures of two MoAbs did not give superior tumor localization as compared with corresponding doses of the individual MoAbs in all three colorectal cancer models, reflecting the *in vitro* cell binding reactivities; the cocktails of MoAbs simply gave averaged values. The MoAbs showed similar tendencies of biodistribution in normal tissues. These results indicated that mixtures of anti-tumor MoAbs recognizing different antigen molecules do not afford better tumor localization than the use of single MoAbs.

There have been conflicting reports on the use of cocktails of MoAbs.[3, 6, 25, 27, 29] Recently, Sharkey *et al.*[27] and Matzku *et al.*[29] reported that the use of combinations of MoAbs did not improve the tumor localization,

but reduced the tumor uptake compared with that of the best MoAb alone, although they used MoAbs directed against different epitopes on the same antigen molecule, CEA or high-molecular-weight melanoma-associated antigen. In contrast, Munz *et al.*[25] reported improved radioimmunoimaging of human tumor xenografts by the use of a mixture of $F(ab')_2$ fragments of MoAbs GA73-3 and CO29.11, as determined by external scintigraphy. An equal mixture of ^{131}I-labeled $F(ab')_2$ fragments recognizing CA 19-9 and CEA has been employed clinically to increase the sensitivity of detection of recurrence of colorectal cancer, as compared with the use of either MoAb alone.[3] However, our results acquired by using cocktails of any two of 19-9, 17-1A and anti-CEA MoAbs were discouraging, irrespective of whether absolute tumor uptake or tumor-to-normal tissue ratios were used as parameters for the comparison.

We have examined the effect of cocktails of anti-tumor MoAbs using nude mice transplanted with colorectal cancer cells of relatively homogeneous character. Human tumors are generally considered to be composed of heterogeneous populations of cells with different antigen expression. Cocktails of MoAbs could not increase the tumor uptake even in Co-3 tumors composed of more than two cell populations expressing different antigens. However, cocktails of labeled MoAbs recognizing different antigens may extend the tumor recognition spectrum. Combinations of MoAbs would be favorable in tumor-bearing patients where antigen expression may be heterogeneous within a lesion and among lesions in different anatomic sites, where the antigen expression in the tumor to be targeted may be limited, or where one MoAb may have some interaction with another MoAb.

The results of clinical studies using radiolabeled anti-tumor MoAbs have shown that a considerable number of problems remain to be solved before scintigraphic localization and therapy using MoAb-conjugates can be optimized. Tumor uptake and tumor-to-normal tissue ratios must be high to deliver effective doses of radionuclides, toxins or anti-cancer drugs selectively to cancer tissues. However, the present studies clearly demonstrate that a cocktail of MoAbs recognizing different antigens does not enhance the tumor uptake of MoAbs above the level given by the corresponding dose of the best single MoAb alone in tumor-bearing nude mice. The selection of a suitable MoAb among many kinds of MoAbs with different specificities generated by the hybridoma technique still remains an important point. MoAb-conjugates which show a high tumor-to-normal tissue ratio when administered *in vivo* are highly desirable for successful tumor targeting, and those patients in whom a high localization can be achieved in scintigraphic studies will be good candidates for therapy using MoAb-conjugates.

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