Iodine-131-Labeled MAb F(ab')$_2$ Fragments Are More Efficient and Less Toxic Than Intact Anti-CEA Antibodies in Radioimmunotherapy of Large Human Colon Carcinoma Grafted in Nude Mice

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During one week, beginning 18 days after transplantation, nude mice bearing human colon carcinoma ranging from 115 to 943 mm^3 (mean 335 mm^3) were treated by repeated intravenous injections of either iodine-131-(^{131}I) labeled intact antibodies or ^{131}I-labeled corresponding F(ab')$_2$ fragments of a pool of four monoclonal antibodies (MAbs) directed against distinct epitopes of carcinoembryonic antigen (CEA). Complete tumor remission was observed in 8 of 10 mice after therapy with F(ab')$_2$ and 6 of the animals survived 10 mo in good health. In contrast, after treatment with intact MAbs, tumors relapsed in 7 of 8 mice after remission periods of 1 to 3.5 mo despite the fact that body weight loss and depression of peripheral white blood cells, symptoms of radiation toxicity, and the calculated radiation doses for liver, spleen, bone, and blood were increased or equal in these animals as compared to mice treated with F(ab')$_2$.

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Radioimmunotherapy using antibodies labeled with isotopes such as iodine-131 (^{131}I) or yttrium-90 (^{90}Y), which emit medium- to high-energy beta-particles, offers the theoretical advantage that these electron particles can penetrate and damage several cell layers within a solid tumor. Thus, due to the crossfire of the beta-radiation, cells expressing low amounts of antigen or cells which are inaccessible to antibodies can be lethally hit by electrons emitted from distant monoclonal antibodies (MAbs). Polyclonal antibodies and MAbs carrying such isotopes have been used in clinical tumor therapy trials of hepatocarcinoma, melanoma, colon carcinoma, and lymphoma (*1–9*).

We and others have previously shown that iodine labeled F(ab')$_2$ fragments of MAbs injected in tumor bearing nude mice can give higher tumor-to-normal tissue ratios than their intact antibody counterparts (*10–13*). F(ab')$_2$ fragments may therefore offer an advantage over intact antibodies for radioimmunotherapy (*14–17*). Another theoretical advantage of F(ab')$_2$ fragments is the absence of the Fc portion, which might lead to decreased radioactivity accumulation in the reticuloendothelial system.

We have evaluated the therapeutic value of a pool of ^{131}I-labeled MAb F(ab')$_2$ in nude mice bearing large human colon carcinoma and compared its efficiency with that of pooled, corresponding ^{131}I-labeled intact MAbs.

METHODS

Monoclonal Antibodies

Intact antibodies and F(ab')$_2$ fragments of four MAbs (MAb 35, CE25-B7, B17, and B93) (*18, 19*) directed against four independent epitopes of carcinoembryonic antigen (CEA) (epitopes Gold 1–4, (*20*)) have been selected according to criteria required for therapeutic injection of patients (*21–23*). These criteria include: high binding rate to purified CEA and *no* binding to:

1. Cross-reacting antigens NCA-55 and NCA-95 (*24*).
2. Biliary glycoprotein (*25*).
3. Fresh human granulocytes (*26*).

The ^{131}I-labeled individual antibodies localize equally well within human colon carcinoma xenografts yielding high tumor-to-whole body ratios ranging from 25 to 40 and from 35 to 50 for intact MAbs and F(ab')$_2$, respectively (*27*).

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Preparation of Intact MAbs and F(ab')$_2$ Fragments

The intact four MAbs (all of the IgG_1 subclass) or normal IgG_1 (n.IgG) were prepared from ascites of the four hybridomas or of the IgG_1-secreting mouse myeloma P3×63 by precipitation with ammonium sulfate. Redissolved IgG was purified by DE 52 (Whatman, Balston, UK) ion-exchange chromatography. F(ab')$_2$ fragments were obtained from ascites by ammonium sulfate precipitation, digestion of the redissolved sediment with pepsin (Sigma, St. Louis, MO) and purification by chromatography on Sephadex G-150 (Pharmacia, Uppsala, Sweden) and DE 52 ion-exchange column (*27*). Purified antibodies (intact and F(ab')$_2$) gave a homogeneous band on SDS-polyacrylamide gel electrophoresis (polyacrylamide 10%, (*28*)) including >95% of the proteins. For therapy and for time-course studies of tissue distribution, the four MAbs were used as a pool, mixed together in equal amounts, either in intact form or as F(ab')$_2$.

Labeling of MAbs and F(ab')$_2$ Fragments

Batches of 1 mg protein of the four pooled anti-CEA MAbs or fragments or of control n.IgG were labeled by the chloramine T method using 10 mCi of ^{131}I, yielding a specific activity of 8–9 μCi/μg. The immunoreactivity of radiolabeled intact MAbs and F(ab')$_2$ was determined as described earlier (*27*): In the direct binding assay of radiolabeled MAbs to CEA insolubilized on CNBr-Sepharose (Pharmacia) intact MAbs bound to 71.8% ± 6.8% and F(ab')$_2$ fragments to 73.6% ± 6.0%. Nonspecific binding of n.IgG and F(ab')$_2$ was <2%. Analytical chromatography of ^{131}I-labeled proteins on Sephadex G-200 gave a single peak without detectable amounts of aggregates.

Nude Mouse Tumor Model

The human colon carcinoma T380 was serially transplanted subcutaneously into the right flank of 7–9-wk-old male "Swiss" homozygous nu/nu mice (Iffa Credo, L'Arbresle, France). Tumor T380 contains almost no necrotic areas up to large tumor sizes, is moderately differentiated and contains numerous pseudolumina which are rich in CEA. From 1 g of tumor ~40 μg CEA can be extracted (*29*).

Mice were kept in aseptic conditions using filter paper topped cages and fed with standard, vitamin-supplemented, irradiated food. In addition, drinking water was supplemented with a polyvitamin preparation (Protovit iv, Roche, Basel, Switzerland, 0.3 ml/300 ml during 4 days of each second week). Lugol iodine solution (5%) was added into drinking water (0.2 ml/300 ml) 3 days before and up to 6 wk after injection of ^{131}I-labeled proteins.

Eight to 10 days after inoculation of minced T380 tumor (50 mm^3) ~90% of the transplants started to grow exponentially and were selected for therapy.

Tumor Size Influence on Antibody Localization

It has been shown that the percentage of injected dose of antibody localization per gram of tumor is inversely related to the size of tumor when ^{111}In-labeled antibodies are used (*30*). We have used ^{131}I labeled anti-CEA MAb F(ab')$_2$ to verify if this rule is also true in our model.

One hundred micrograms of anti-CEA MAb F(ab')$_2$ together with 1.5 μCi ^{131}I-labeled MAb F(ab')$_2$ were injected intravenously into mice bearing colon carcinoma T-380 tumors of different size. Eight hours after injection, when maximal percentage of tumor localization was reached (Ref. *14* and results shown here), the mice were killed and radiolabeled antibody distribution was measured in tumor and normal tissues and expressed in percentage of injected dose per gram (% ID/g). Tumor localization data were plotted against tumor size and then compared with different equations on a curve-fitting program.

Therapeutic Injection of ^{131}I-MAbs, F(ab')$_2$ and Control Proteins

Eighteen days after tumor transplantation (tumor T380, passage 52), 30 animals bearing exponentially growing tumors (transplanted the same day with the same inoculum) were randomly distributed in five groups except for the six mice with largest tumors which were distributed into the two therapy arms for being injected with either ^{131}I-intact MAbs or ^{131}I-MAb F(ab')$_2$. The injected baseline ^{131}I radioactivity was selected in order to deliver similar radiation doses to each tumor. Our previous experiences (*14*, *27*) and further preliminary results had shown that we needed four to five times more ^{131}I F(ab')$_2$ than intact antibodies. In addition, since the percentage of ID/g of tumor decreases with increasing tumor size (see Fig. 1), we had to adapt injected radioactivity for each mouse following the formula shown below.

For ^{131}I-intact MAb injections, a baseline 500-μCi dose was supplemented with 0.25 μCi/mg tumor weight (TW) according to:

$$\text{Total activity intact MAb (in } \mu\text{Ci)} = 500 + 0.25 \times \text{TW (in mg)}.$$

Two-thirds of this dose was injected on Day 18 and one-third on Day 25 after tumor transplantation.

For ^{131}I-MAb F(ab')$_2$ injections, the formula was similarly chosen according to:

$$\text{Total activity F(ab')}_2\text{ (in } \mu\text{Ci)} = 2{,}200 + 1.1 \times \text{TW (in mg)}.$$

This amount was split in three identical doses injected on Days 18, 20, and 25.

For both injections of intact MAbs and F(ab')$_2$ by using these formulas, the injected dose was increased by 50% for mice bearing 1-g tumors as compared to mice with very small tumors. All the fractionated doses were injected within 1 wk because in patients development of human anti-mouse IgG antibodies (HAMA), often limits this time period.

The activities of ^{131}I were calculated for mice of 30-g body weight. For smaller and heavier animals, the amount was corrected according to weight difference from 30 g.

Four control mice were injected with ^{131}I-n.IgG F(ab')$_2$ together with 300 μg unlabeled anti-CEA MAb F(ab')$_2$. The amount of microcuries of ^{131}I and of microgram proteins was calculated as it was for ^{131}I-MAb F(ab')$_2$, and was injected in three doses at the same times.

Three control mice were injected with ^{131}I-intact n.IgG together with 75 μg unlabeled intact anti-CEA MAb, as in ^{131}I-intact MAb injections. It also was given in two doses. This control group exhibited the strongest bone marrow depression (very low peripheral white blood cells (pWBCs) and hemoglobin) and two mice died shortly after BMC transplantation. In the third mouse, the tumor relapsed rapidly after a short remission period (data not shown).

The last control group consisted of five tumor-bearing animals which were not injected.

Tumor-Size Measurements

Three diameters of the tumors (which can be easily moved around subcutaneously), were measured twice a week for 50 days and then once a week. Tumor volume (V) was calculated by:

$$V = {}^4/_3 \pi \times r_1 \times r_2 \times r_3,$$

where r is the tumor radius.

The accuracy of these measurements was ±10%–15% when performed by different observers and by comparing results obtained by external tumor measurements and direct weighing of 20 dissected tumors.

Toxicity Evaluation in Antibody Treated Mice and Controls

Whole-body counting was performed immediately after injecting ^{131}I-intact antibodies and F(ab')$_2$ and every 1 to 2 days thereafter using a dose calibrator (Isotope Assayer 1, RADX, Houston, TX).

On the day of injection and every 2 to 3 days thereafter, mice were weighed until they recovered from initial weight loss.

On Days 7, 14, 19, 22, 27, and 43 after beginning ^{131}I injections, blood was taken from a tail vein and peripheral pWBCs were counted.

Bone Marrow Transplantation

Nude mice with pWBC falling below 1000 cells/mm^3 were transplanted with bone marrow cells (BMC) to prevent microbial infections. Donor nude mice of the same "Swiss" genetic background were killed and BMC washed out from long bones (femur, tibia, and humerus) using a small volume of serum-free culture medium. About 12 × 10^6 BMC were recovered from a donor mouse and 4 × 10^6 BMC were injected intravenously per recipient mouse. None of the mice treated with ^{131}I-anti-CEA MAb F(ab')$_2$ and only one in the group treated with ^{131}I-anti-CEA intact MAbs needed BMC transplantation. In contrast, several control mice injected with ^{131}I-n.IgG in intact form or as F(ab')$_2$ had to be transplanted.

Dosimetry for Tumor and Normal Organs

Calculation of radiation doses for tumor and normal tissues were based on time-course studies of ^{131}I tissue distribution in mice injected with trace amounts of ^{131}I-MAb together with unlabeled antibodies either in intact form or as F(ab')$_2$.

Twenty-seven and 18 mice were injected with 10 μCi ^{131}I-intact anti-CEA MAb or anti-CEA MAb F(ab')$_2$ together with 50 μg unlabeled intact MAb or 100 μg unlabeled MAb F(ab')$_2$, respectively. At different times postinjection (1, 4, 8, 12, 24, 48, 80, 120, and 168 hr) groups of three mice were killed, dissected, and tissue distribution of radioactivity was measured. From the amount of radioactivity measured directly at these timepoints, an integral activity in μCi × h was calculated per gram of tumor and normal tissues. Tissue-absorbed beta-radiation for tumor, normal organs, and whole body was then calculated according to (*31*):

$$D_\beta = 2.13 \times \mu Ci/g \times h \times E_\beta \text{ rad}$$
$$[E_\beta \text{ of } ^{131}I = 0.19 \text{ g}/(\mu Ci \times h)].$$

An additional gamma-radiation was assumed to be equally distributed in the whole animal. The gamma-radiation represents ~10% of the beta whole body radiation for a 30-g mouse (*27*).

A further, comparable time-course study after injecting 18 mice with ^{131}I-MAb F(ab')$_2$ mixed with 270 μg unlabeled MAb F (ab')$_2$ has been published by us (*14*).

Statistical Analysis

Results were analyzed quantitatively and qualitatively using the Student t-test and the χ^2 test, respectively. Weight data and pWBCs measured on different days were also comparatively analyzed by a two-factors-analysis of variance.

RESULTS

Influence of Tumor Size on Antibody Localization

Fifteen mice bearing tumors of different size were injected with 100 μg unlabeled MAb F (ab')$_2$ together with trace amounts of ^{131}I- (1.5 μCi) anti-CEA MAb F(ab')$_2$. Eight hours after antibody injection, the mice were killed and tumor as well as normal tissue radioactivity distribution was measured and expressed in % ID/g. Tumor radioactivity was high in mice bearing small tumors (25%–38% ID/g) and exponentially decreased in mice with larger tumors, reaching 5.4% ID/g in a mouse bearing a 7.9-g tumor (Fig. 1). Best curve fitting was obtained to a line following the equation:

$$Y = \frac{A}{(B + X)},$$

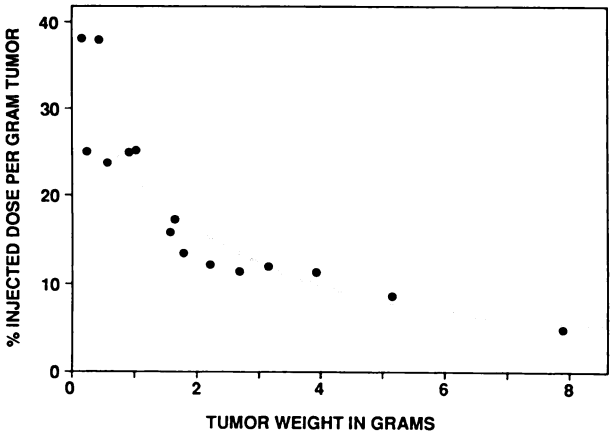


FIGURE 1
Influence of tumor size on antibody localization. Percent injected dose of ^{131}I-anti-CEA MAb F(ab')$_2$ per gram of tumor was determined in 15 nude mice bearing colon carcinoma T380 transplants of different size varying between 0.2 to 7.9 g. One hundred micrograms unlabeled MAb F(ab')$_2$, mixed with 1.5 μCi (2μg) ^{131}I-MAb F(ab')$_2$ were injected intravenously, and tissue distribution in the animals was measured at 8 hr after injection. The percentage of ID/g of tumor (●) is plotted against tumor size. The regression curve is determined by the equation:

$$\%\ ID/g\ tumor = \frac{53.2}{1.44 + \text{tumor weight (mg)}}.$$

where Y is the % ID/g tumor, X being tumor weight in grams, and A and B are constants with values of 53.21 and 1.44, respectively. The coefficient of determination for this regression was very high with r = 0.95. The expected localizations calculated from this equation for infinitely small tumors, tumors of 0.3 g and of 1 g would be 36.9%, 30.6%, and 21.8% ID/g, respectively.

Tumor Size Evolution in Mice Treated with ^{131}I-labeled Anti-CEA MAb F(ab')$_2$ Fragments

Ten mice bearing tumors of different size (ranging from 151 to 943 mm^3, mean 361 mm^3) were treated with three injections of 720–980 μCi each (depending on tumor size), mean 810 μCi, of ^{131}I-labeled F(ab')$_2$ fragments. As observed earlier, all tumors continued to grow for a few days, reaching a maximum size of 529 mm^3 ± 408 mm^3 (range 194–1383 mm^3) (Fig. 2A). Tumor size then decreased in all mice and on Day 84 after therapy an overall minimal size of 105 ± 63 mm^3 (range 48 to 212 mm^3) was observed. In 8 of the 10 mice, tumor remission was complete, with no evidence of relapse. Tumor relapse was observed in two mice ~110 days after therapy. These two relapses occurred in mice with relatively small tumors during treatment. Two of the eight mice with complete remissions died at 6 mo after therapy due to baterial infection (verified by histology at autopsy).

The remaining subcutaneous nodules of the eight mice which had no evidence of tumor relapse after ^{131}I-MAb F(ab')$_2$ therapy were examined histologically (Fig. 3): Three nodules contained only fibrotic and necrotic tissue (Fig. 3A–B), whereas some sparse epithelial tumor cells (without any evidence of mitotic division) embedded in fibrosis, remained present in the other five nodules (Fig. 3C–D).

In order to test whether the animals with complete tumor remission 10 mo post-therapy had developed some form of immune response against the T380 human colon carcinoma, a new T380 tumor graft was transplanted into five mice with apparently sterilized tumors. In four mice, the newly transplanted T380 tumor grew as usual and reached 1000 mm^3 between 25 and 38 days after transplantation. In one mouse, (after some initial growth) the tumor was rejected after 15 days. The secondary take of the T380 tumor xenograft in four out of five animals strongly suggests that the complete remissions observed in ^{131}I-MAb F(ab')$_2$ treated animals was essentially due to the ^{131}I irradiation and not to an immunologic response against the human tumor.

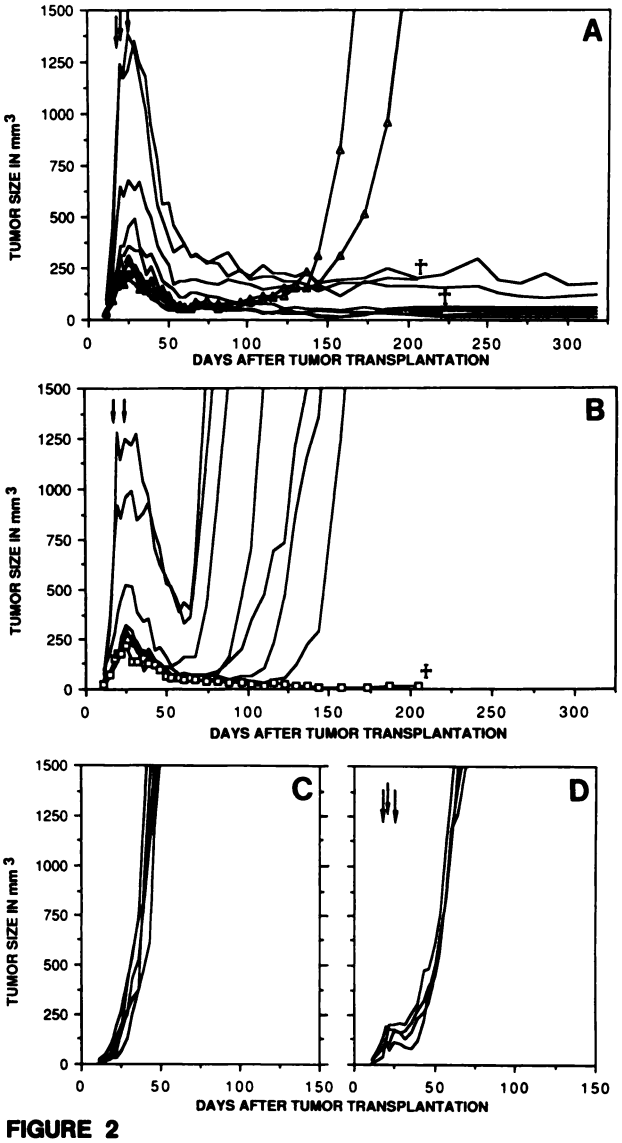
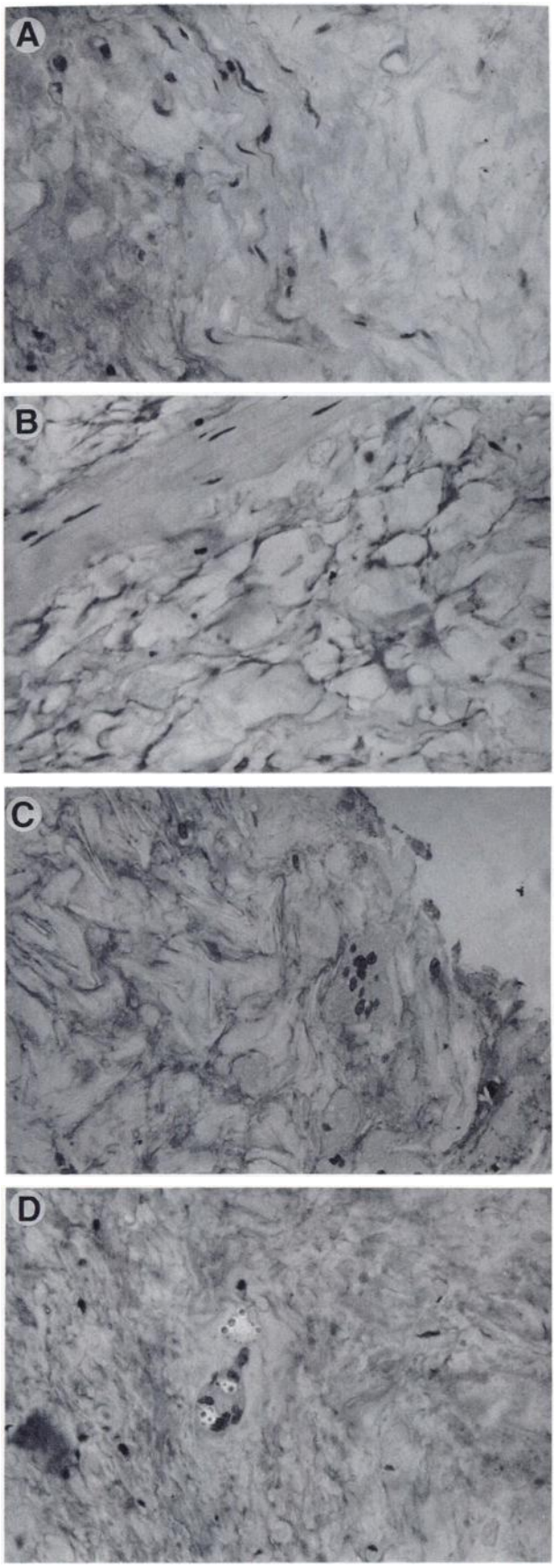


FIGURE 2
Evolution of tumor size in treated mice and controls. (A) Ten mice bearing tumors of 151 to 943 mm^3 (mean 361 mm^3) on Day 18 after transplantation were treated with 3 injections of ^{131}I-MAb F(ab')$_2$ fragments 18, 20, and 25 days (arrows) after tumor transplantation. Tumor regression was observed in all mice. In two mice, tumors relapsed ~4 mo after therapy (△), two mice died from infection without tumor relapse at around 6 mo (†). (B) Eight mice bearing tumors of 115 to 730 mm^3 (mean 304 mm^3) were treated by two injections of ^{131}I-intact MAbs 18 and 25 days (arrows) after tumor transplantation. After initial remission, tumor relapse was observed in seven of eight mice 4 to 15 wk after therapy. Only one mouse showed no tumor relapse (□) and survived for 6 mo after therapy, when it died (†). (C) In five animals, tumor evolution was observed without treatment. (D) In four other control mice injected with three doses (arrows) of 800 μCi ^{131}I-normal IgG F(ab')$_2$ together with 100 μg unlabeled anti-CEA MAb F(ab')$_2$, tumor growth was retarded for ~3 wk.

Tumor Size Evolution in Mice Treated with ^{131}I-labeled Intact Anti-CEA MAbs

Eight mice bearing tumors of different size (ranging from 115 to 730 mm^3, mean 304 mm^3) were treated with 485–700 μCi (depending on tumor size, mean dose 564 μCi) of ^{131}I-labeled intact MAbs injected in two doses. All tumors continued to grow reaching a maximum size of 506 ± 362 mm^3 on Day 7. Tumor size then decreased (Fig. 2B) and an overall minimal tumor



size of 151 ± 141 mm^3 was measured 42 days after starting therapy. Remission lasted from 1 to 3.5 mo, then seven of eight tumors relapsed. The time of relapse depended on the initial tumor size during therapy, larger tumors relapsing earlier than smaller ones (Fig. 2B). Only one mouse remained in complete remission and in apparent good health for 6 mo. It then died, probably from an infectious disease (infectious agent not determined).

The difference in the number of mice without tumor relapse 6 mo after therapy with ^{131}I-MAb F(ab')$_2$ (8 of 10 mice), and with ^{131}I-intact MAbs (1 of 8 mice) was highly significant ($p < 0.005$).

Tumor Size Evolution in Control Mice

Five mice transplanted with colon tumor T380 on the same day as treated animals were observed without any treatment (Fig. 2C). The 5 tumors grew rapidly and reached 2 g between Days 45 and 57 (mean 50 days).

Four other control mice bearing tumors of 113–202 mm^3 were injected with three equal doses of ~800 μCi ^{131}I-n.IgG F(ab')$_2$, together with 100 μg of the unlabeled anti-CEA MAb F(ab')$_2$ (Fig. 2D). In these animals, tumor growth was retarded by ~3 wk. All four tumors reached 2 g between Days 64 and 81 (mean 71) after transplantation.

Three additional tumor-bearing mice were injected on Days 18 and 25 after transplantation, with a total of ~530 μCi ^{131}I-intact n.IgG and 75 μg of the pooled four unlabeled anti-CEA MAbs. Toxic side effects in these three animals were very important and two animals died after bone marrow transplantation. Tumor growth in the remaining animal was retarded by ~30 days (data not shown).

Side Effects of Treatments with ^{131}I-intact MAbs and ^{131}I-F(ab')$_2$ Fragments

Side effects of treatment with ^{131}I-labeled intact antibodies as well as with ^{131}I-F(ab')$_2$ fragments included weight loss and bone marrow depression, the latter being detected through decrease of pWBC counts.

Weight loss after treatment with intact antibodies was most pronounced four days after the second injection when it was 8% (range from 3% to 11%) (Fig. 4A). After treatment with F(ab')$_2$ fragments, weight loss was ~4% (range 0%–7%) four days after the last injection.

FIGURE 3
Histology of tumor nodules remaining after treatment with F(ab')$_2$ fragments. Histologic sections of 3 μm from nodules of eight mice without tumor relapse 10 mo after treatment with ^{131}I-MAb F(ab')$_2$ are shown. Absence of tumor cells was observed in three nodules, where only fibrosis, necrosis, and some muscle cells were seen, illustrated by images A and B. In C and D, a single group of epithelial like cells embedded in large fibrosis is shown, representative of the other five nodules. Such epithelial cells were rare in the large areas of fibrosis and they showed no evidence of cell division.

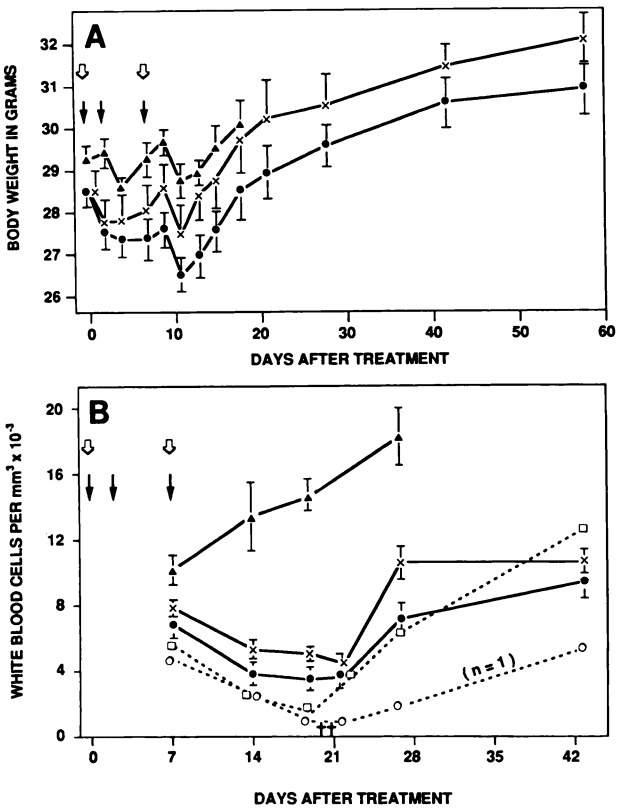


FIGURE 4

Comparison of weight loss and peripheral WBC counts in the therapy groups and in untreated animals. All mice were weighed immediately before and every 2 to 3 days after therapy (A). Weight loss was more pronounced in mice treated with intact antibodies (mean loss 8%), (⇓, ●) as compared to mice treated with F(ab')$_2$ (mean loss 4%), (↓, ×). Untreated animals also showed some weight variations, but less pronounced (▲). At 7, 14, 19, 22, 27, and 43 days after the beginning of therapy, pWBC were counted (B). Mice injected with ^{131}I-intact MAbs (⇓) are indicated by (●); those injected with ^{131}I-F(ab')$_2$ fragments (↓) by (×). Decrease of pWBC in mice treated with intact antibodies was more pronounced as compared to mice treated with F(ab')$_2$. In untreated, tumor-bearing control mice, pWBCs were increasing during the observation time (▲). Decrease of pWBC in mice injected with ^{131}I-intact n.IgG (○) and ^{131}I-n.IgG F(ab')$_2$ (□) was more pronounced as compared to anti-CEA antibody treated animals. Two mice injected with intact n.IgG were lost (⊕) after bone marrow transplantation, which had to be given to several animals from the control groups because of a very low pWBC. The variance of pWBC measurements in the small control groups was relatively high (~30% of the mean). For the larger antibody therapy groups, vertical bars show the s.e.m.

Weight loss in mice treated with ^{131}I-labeled intact MAbs and with F(ab')$_2$ fragments was statistically compared. Mean body weight was identical in the two groups at the beginning of therapy. Weight loss was more marked in mice treated with intact antibodies already after the first injection. The difference became significant after 7 days ($2p < 0.02$) and remained significant thereafter for all the measurements shown in Figure 4A. The two-factors-analysis of variance, considering all weight data from 7 up to 57 days after treatment (total of 180 weight measurements), indicated a highly significant difference ($p < 0.0002$).

Peripheral WBC reached its lowest values at Days 14, 19, and 22 after beginning of therapy in both groups (Fig. 4B). The values observed on these days should be compared with the increasing values in tumor transplanted mice which were not treated. From all treated mice, only one animal in the group injected with intact antibodies required bone marrow transplantation because pWBC fell below 1000 cells/mm^3. The lowest value measured in mice treated with fragments was 2500 cells/mm^3.

As shown for weight loss, it appears that the decrease in pWBC counts was also more pronounced after treatment with ^{131}I-intact MAbs as compared to fragments. A two-factors-analysis of variance comparing all data shown in Figure 4B (total of 108 pWBC measurements) was highly significant ($p < 0.0005$). However, the pWBC counts at the start of therapy are not known (they should, in principle, be similar in the two randomized groups). We, thus, conclude that the higher doses of ^{131}I-F(ab')$_2$ were, at least, not more toxic than intact MAbs.

Hemoglobin also was decreased in treated animals as compared to untreated mice. This decrease was much less marked than that of pWBC. Hemoglobin was decreased to 78% ± 11% after treatment with intact antibodies and to 88% ± 4% after treatment with F(ab')$_2$ fragments.

Side Effects in Control Mice Injected with ^{131}I-n.IgG Together with Unlabeled MAb and Comparison to Uninjected Mice

In uninjected, tumor-bearing mice, some variation in the weight was observed (Fig. 4A). The pWBC of these animals increased continuously from 10.1×10^3 cells/mm^3 (range 8.4 to 12.9×10^3) on Day 25 after transplantation, to 18.3×10^3 cells/mm^3 (range 15.2–22×10^3) on Day 44 (Fig. 4B). Mice kept in the same facility without transplanted tumors had 8.3×10^3 pWBC/mm^3 (range 3.3–15.6×10^3, n = 8).

In mice injected with ^{131}I-n.IgG F(ab')$_2$ together with unlabeled anti-CEA MAb F(ab')$_2$, weight loss and pWBC decrease were slightly more pronounced as compared to mice treated with ^{131}I-anti-CEA MAb F(ab')$_2$. The higher toxicity of ^{131}I-n.IgG F(ab')$_2$ correlates with a longer whole-body half-life of radioactivity as compared to mice bearing tumors of similar size treated with ^{131}I-anti-CEA MAb F(ab')$_2$ (data not shown).

In mice injected with ^{131}I-intact n.IgG and unlabeled anti-CEA MAb, toxicity was very high as illustrated by weight loss (~7%), and particularly pWBC counts that fell below 1000 cells/mm^3 in all three mice. Low pWBC were paralleled by very low hemoglobin values (38%–58% when compared to untreated animals). Two of these mice died shortly after BMC transplantation,

concomitant with the lowest measured hemoglobin values of 38% and 41%.

Dosimetry

Time-course studies of the biodistribution of ^{131}I-labeled anti-CEA MAb F(ab')$_2$ (pool of the four antibodies, Fig. 5A) and of the corresponding intact MAbs (Fig. 5B) were performed on series of 18 and 27 mice, respectively, bearing T380 tumor grafts of an average size of 0.26 ± 0.11 g (range 0.11–0.44 g) and of 0.37 ± 0.15 g (range 0.1–0.67 g), respectively. Tissue distribution of radioactivity in tumor and normal organs at different times after injection allowed calculating the μCi × hr/g of each organ, which, in turn, was converted into radiation doses as described in the Methods section.

The injected ^{131}I-MAb F(ab')$_2$ localized rapidly in the colon tumor T380 transplants and reached ~32% ID/g after 8 hr (Fig. 5A). After 12 hr, tumor radioactivity decreased with a T/2 of ~1 day. The high percentage of ID/g tumor obtained after injection of F(ab')$_2$ fragments may appear to be in conflict with earlier published data (*13*). This is due to the fact that in the earlier work, tissue distributions of F(ab')$_2$ have been analyzed 2–3 days after injection, while here the entire kinetics of MAb tissue distributions have been measured. Similarly, as for F(ab')$_2$, intact antibodies reached a maximal tumor localization of ~32% ID/g after 24 hr. Decrease of tumor radioactivity of intact MAbs after 24 hr was longer with T/2 of ~3 days.

In normal tissues, after injection of ^{131}I-MAb F(ab')$_2$ fragments, percentage of ID/g decreased rapidly to nearly 0% at 48 hr, while after injection of ^{131}I-intact MAbs a significant percentage of ID/g remained after 7 days (Fig. 5A–B).

For normal tissues, we considered that the radiation doses calculated for one injection could be extrapolated to two or three injections by simple multiplication, since the whole-body half-lives measured after repeated injections in animals without tumor or with tumors below 0.2 g were almost identical after each injection (data not shown). For tumor tissue, however, extrapolation for repeated injections was not as clear and was thus more difficult to determine: The percentage of ID/g tumor could be lower after the second and third injections due to beginning necrosis and partial saturation of antigen by MAb remaining from previous injections.

In mice bearing large tumors (0.5–1.5 g) the whole-body half-life of F(ab')$_2$ was markedly influenced by the retention of antibody within the tumor. This allowed us to calculate that the tumor uptake of antibody was reduced by ~30% after the second and third injections as compared to the first one. Thus, we have calculated total tumor radiation doses for mice injected both with ^{131}I-intact antibodies and ^{131}I-MAb F(ab')$_2$, by reducing by 30% the dose of radioactivity delivered by the second and third injections, as compared to the first one. The tumor radiation doses of 9,000 rad (Table 1) should be considered with some reservation because of this approximation. The comparison of irradiation of normal tissues by intact antibodies and fragments, however, remains absolutely valid.

After injecting relatively high doses of ^{131}I-fragments, normal mouse tissues received between 190 rads (muscle) and 2,220 rads (blood), mean whole-body dose being 440 rads. After injecting 4.5 times smaller doses of ^{131}I-intact MAbs, radiation doses to normal tissue were comprised between 300 rads (muscle) and 3,120 rads (blood), the mean whole-body dose being 660 rads (Table 1). Thus, several tissues have been exposed to higher levels of irradiation after injecting intact antibodies as compared to fragments. Among different

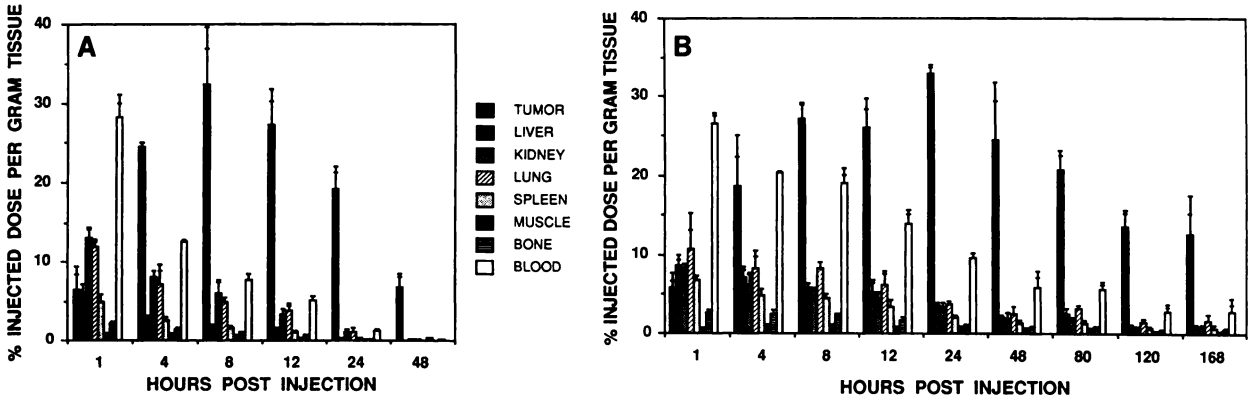


FIGURE 5

Time course study of tissue distributions of ^{131}I-labeled MAb F(ab')$_2$ and intact MAbs. Results of two series of mice are shown which were injected either with (A) 10 μCi ^{131}I-anti-CEA MAb F(ab')$_2$ mixed with 100 μg unlabeled MAb F(ab')$_2$ (n = 18) or (B) with 10 μCi ^{131}I-intact anti-CEA MAb together with 50 μg unlabeled MAb (n = 27). Groups of three mice were killed at different times after injection starting at 1 hr up to 48 hr for F(ab')$_2$ and up to 168 hr for intact MAbs. The mice were dissected and tissue distribution of radioactivity was measured and expressed in % ID/g. The different tissues shown are, from left to right: Tumor, liver, kidney, lung, spleen, muscle, bone, and blood. Columns represent calculated mean values of %ID/g, while the vertical lines, cut by two horizontal lines, represent the upper and lower ranges.

TABLE 1
Tumor and Normal Tissue Absorbed Radiation Doses (in rads) Estimated from Biodistribution Studies

	^{131}I-F(ab')$_2$	^{131}I-intact MAbs	
Tumor	9170*	9420*	
Blood	2220 (4.1)†	3120 (3.0)**	+41%‡
Kidney	1450 (6.3)	1150 (8.2)	−21%
Lung	1400 (6.6)	1580 (6.0)	+13%
Stomach	1040 (8.8)	480 (19.6)	−54%
Liver	580 (15.8)	1120 (8.4)	+93%
Spleen	510 (18.0)	790 (11.9)	+55%
Bone	310 (29.6)	500 (18.8)	+61%
Small intestine	340 (26.9)	400 (23.6)	−18%
Large intestine	220 (41.7)	250 (37.7)	−14%
Muscle	190 (48.3)	300 (31.4)	+58%
Whole mouse	440 (20.8)	660 (14.3)	+50%

* Tumor and normal tissue irradiations are given in rads.

† Tumor-to-normal tissue irradiation ratios are indicated in parentheses.

‡ Percent increase/decrease of irradiation for normal tissues after injection of ^{131}I-intact antibodies as compared to irradiation after injection of ^{131}I-F(ab')$_2$ fragments.

tissues the increase for bone was 61%, for blood 41%, for spleen 55%, and for liver 93% (Table 1). Conversely, radiation exposure after injecting F(ab')$_2$ was 116% higher for stomach and 26% higher for kidneys as compared to intact MAbs.

Radioactivity localized in bone marrow was not measured directly. However, irradiation doses calculated for bones, blood, and spleen were definitively lower after injecting fragments as compared to intact MAbs. This strongly suggests that bone marrow received less irradiation when fragments were used.

DISCUSSION

We have previously shown that nude mice bearing small human colon carcinoma transplants could be cured by i.v. injections of ^{131}I-labeled anti-CEA MAb F(ab')$_2$ fragments (*14*). These results were encouraging but they had been obtained by starting treatment on Days 9 to 10 after transplantation when the colon carcinoma T380 xenografts, albeit in exponential growth, were still relatively small (mean maximal size: 102 ± 50 mm^3).

In the present experiment, therapy was begun 18 days after transplantation, when the tumors were much larger, allowing us to confirm the therapeutic effectiveness of ^{131}I-anti-CEA MAb F(ab')$_2$ fragments on mice bearing tumors which reached a mean maximal size of 530 mm^3 (range 190 to 1400 mm^3). Complete remissions without tumor relapse were observed in 8 of 10 mice and this does not appear to be due to an immunologic rejection, since the majority of successfully treated mice readily accepted a subsequent challenge with the same human colon carcinoma xenograft. The low toxicity of high doses of ^{131}I-MAb F(ab')$_2$ was confirmed by the fact that in these animals pWBC never fell below 2,500 cells/mm^3 and none needed bone marrow transplantation. Furthermore, weight loss during treatment was minimal and except for two mice which died from infection at 6 mo post-therapy, no other side effects developed during 10 mo of observation.

Several reports have described delayed tumor growth and, in some cases, complete remissions of solid tumor transplants by treatments using various radiolabeled MAbs (*15, 16, 32–37*). Complete remissions, however, have often been obtained by initiating antibody therapy within 24 hr after tumor transplantation, or, in other cases, by injecting very large amounts of radiolabeled antibodies, which caused severe radiation toxicity and death of a high number of animals (*34*).

Recent efforts are therefore directed toward reducing bone marrow toxicity from radiolabeled MAbs. An interesting approach is the injection of Interleukin-1 before and/or during radioimmunotherapy (*38*). Injection of biotinylated, radiolabeled antibodies followed by a secondary injection of avidin has been proposed as a mean to provoke a rapid clearance of circulating antibodies (*39*). Along similar lines, a secondary injection of anti-mouse-IgG antibodies had been given to produce a more rapid blood clearance of radiolabeled first antibodies (*5, 40*). It has also been proposed to inject unlabeled antibodies prior to radiolabeled MAb in order to reduce liver uptake of radiolabeled antibody (*41*).

A more direct manner of decreasing marrow and liver uptake of radiolabeled antibody is to use fragments of MAbs. F(ab')$_2$ and Fab fragments give higher tumor-to-normal tissue ratios in experimental tumor models (*10–13*) and are widely used for immunoscintigraphy in patients (*2, 22, 23*). Very few experimental results, however, have been published on their use for therapy. Our recent results (*14*) and the present study demonstrate experimentally the superiority of F(ab')$_2$ over intact MAbs in radioimmunotherapy. These results are in agreement with those obtained in a model of colon tumor transplants in hamsters (*16*), with a dosimetric study performed in a glioma xenograft model in nude mice brains (*15*), and with a rat model system (*17*). These three studies too compared MAb F(ab')$_2$ tissue distributions with the corresponding intact antibodies. Larson et al. made a logical choice when they used radiolabeled fragments (Fab) for radioimmunotherapy of melanoma patients (*2*). In our hands, however, the half-life of Fab fragments from anti-CEA MAbs is too short to deliver sufficient amount of radioactivity to the tumor.

Here, in matched groups of tumor-bearing nude mice, we essentially show that i.v. injections of large doses of ^{131}I-MAb F(ab')$_2$ give a much better therapeutic

efficiency together with similar or reduced general toxicity, as compared to ^{131}I-intact MAbs. The lower toxicity of F(ab')$_2$ fragments is in agreement with the reduced radiation doses calculated for vital organs such as liver, spleen, bone, and blood when compared to treatment with intact antibodies. Due to the difficulty of calculating tumor radiation doses after repeated injections, the value of ~9,000 rad, both for treatment with intact MAbs and F(ab')$_2$ fragments, represents an approximation. The superior anti-tumor effect of F(ab')$_2$ (as compared to intact MAbs) could be due therefore to at least three factors:

1. The real tumor dose obtained after injection of fragments could be slightly higher than that obtained with intact MAbs.
2. A deeper penetration of F(ab')$_2$ is likely to have produced a more homogenous distribution of radiation within tumor tissue, as shown earlier by autoradiography (*13*). The fact that larger tumors treated with intact antibodies relapsed more rapidly (Fig. 2B), while this was not the case with F(ab')$_2$, supports this hypothesis.
3. Due to their very short biologic half-life, the radiation dose was delivered by the high amount of ^{131}I F(ab')$_2$ within a shorter period of time and with higher energy flux (peak localizations in tumor reached 200–280 μCi/g after the three injections) than after injection of the lower amounts of ^{131}I-intact antibodies (peak localizations were ~120 μCi/g tumor). This may have allowed less DNA repair to occur within the tumor after injection of F(ab')$_2$.

Our experimental results with ^{131}I-labeled MAb F(ab')$_2$ demonstrate the feasibility of successful radioimmunotherapy of large colon carcinoma xenografts. These results, however, cannot be directly extrapolated to clinical therapy because: (a) human bone marrow is known to be 2–3 times more sensitive to ionizing radiation as compared to murine marrow, and (b) the presence of some CEA in normal human colon mucosa (*42*), results in a slightly increased localization of anti-CEA antibodies in this tissue as compared to others (*22, 23*) (the clinical relevance of this accumulation remains unclear).

Our clinical experience in treating liver metastases of colon carcinoma by injecting large doses of ^{131}I-anti-CEA MAb in intact form and as F(ab')$_2$ fragments is indeed limited, and we have not yet obtained significant tumor remission (*7*). Theoretically, the absolute amount of injected antibody per gram of tumor in patients is expected to be ~2,000 times lower than that in mice as a consequence of the different body weights (*43*). In contrast, tumor-to-normal tissue radioactivity ratios could be similar in mice and men. Indeed, our results in patients indicate, that this is the case: This is illustrated by measurements of the % ID/g in early (at 24 hr after injection) resected tumors of patients reaching 0.02 percent (*23*), and in some favorable cases 0.03% and 0.06% (personal observations), while the calculated mean whole-body retention at 24 hr was ~0.001% ID/g (for a 70.000-g patient with a whole-body ^{131}I retention of 70%). Bone marrow toxicity remains thus the major dose-limiting side effect in patients. This toxicity might be overcome by autologous bone marrow transplantation.

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