Cure of Metastatic Human Colonic Cancer in Mice with Radiolabeled Monoclonal Antibody Fragments

Thomas M. Behr, Rosalyn D. Blumenthal, Stavros Mentzoudis, Robert M. Sharkey, Stefan Gratz, Wolfgang Becker, and David M. Goldenberg

Department of Nuclear Medicine of the Georg-August-University, Göttingen, Germany [T. M. B., S. M., S. G., W. B.], and Garden State Cancer Center, Belleville, New Jersey 07109 [R. D. B., R. M. S., D. M. G.]

ABSTRACT

There is currently no method to cure patients with disseminated colorectal cancer, which is the third leading cancer killer in the Western World. This report shows that the GW-39 intrapulmonary micrometastatic human colonic cancer model in nude mice can be cured with radiolabeled antibodies against carcinoembryonic antigen, and that this approach of radioimmunotherapy is superior to conventional chemotherapy with 5-fluorouracil and leucovorin (5-FU/LV). Monovalent Fab fragments labeled with $^{131}$I are superior to intact IgG when survival was evaluated 3, 7, and 14 days after implantation, leading to cures in up to 90% of the mice. Histological results provide support for the differences in therapeutic efficacy observed. Microautoradiography was used to evaluate the intratumoral distribution of each form of antibody. The enhanced tumor control by Fab compared with IgG could be explained in part by the homogeneity of radioantibody distribution of Fab. Biodistribution analysis and initial dose rate calculations for all three forms of antibody also help explain the ability of $^{131}$I-labeled Fab to provide better tumor growth control than seen with $^{131}$I-labeled IgG. Thus, radioimmunotherapy may be a new modality to treat metastatic disease, particularly when using small antibody fragments.

INTRODUCTION

Despite improvements in the surgical management of cancer, the prognosis of patients with solid tumors has not improved significantly over the past decades (1). For example, in colorectal cancer, which is the third most frequent malignancy in both sexes, 60% of patients will develop local tumor recurrence or distant metastases (2, 3). At the time of primary surgery, tumor cells have been found in the bone marrow of >30% of colorectal cancer patients (4–6). Although the skeleton is not a preferred site of overt metastasis in this disease, the presence of tumor cells here is interpreted as evidence of the tumor’s general disseminative capacity and a strong predictor of later clinical relapse (7). Adjuvant therapeutic strategies aim at killing these residual cancer cells to increase the relapse-free survival period. Indeed, Moertel et al. (8) showed that a combination regimen of 5-fluorouracil and levamisole increases the relapse-free 5-year survival by ~30%. Similar results have been reported for 5-fluorouracil-folinic acid combinations (9, 10), which are most frequently used for treating clinically apparent metastatic disease as well (11, 12). More recently, an immunotherapeutic approach with the monoclonal antibody CO17-1A, which is a murine intact IgG2a directed against a cell surface-associated 41,000 glycoprotein of colorectal cancer cells, but which is also expressed on normal epithelia (13, 14), has yielded results comparable with those of adjuvant chemotherapy (15). Interestingly, however, although CO17-1A decreased the incidence of distant metastases, it was not able to reduce the incidence rate of local recurrences (15), which was interpreted on the basis that single tumor cells, which are the progenitors of future distant metastases, may be more susceptible to antibody-mediated immunological effector mechanisms than larger cell clusters. These tumor cell clusters, which were left in the surroundings of the previous primary tumor and from which local recurrences arise, may be less amenable to penetration by the intact antibody or infiltrating host effector cells (16, 17).

In this context, radioimmunotherapy, involving the use of anticancer antibodies conjugated with therapeutic radionuclides, appears as an attractive alternative, because cross-fire radiation from cells targeted by the radiolabeled antibody may deliver tumoricidal doses to surrounding cells as well (18). Although results have been disappointing in bulky disease of solid tumors (19), the potential of radiolabeled antibodies to treat micrometastases or minimal residual disease has been observed (20, 21). The advantages of smaller immunonconjugates, such as F(ab)$_2$ fragments, with respect to faster and more homogeneous tumor uptake (because of their higher diffusion capacity and more rapid background clearance), has been recognized for many years (22). Even smaller molecular recognition units, such as Fab fragments or peptides, are generally believed not to be suitable for therapeutic purposes. There are two major reasons for this assumption: (a) their tumor uptake is lower than with bivalent IgG or F(ab)$_2$, possibly resulting in lower radiation doses to the tumor (23); and (b) because of the high renal accretion of small fragments and peptides, below the glomerularly filtrable size ($M_r$ ~60,000), radiation nephrotoxicity may become an important limitation in the therapeutic application of such agents (24, 25).
Because we have recently developed methods to effectively decrease the renal accretion of proteins and peptides (24) and also have provided evidence that higher dose rates obtained with such radiolabeled agents may compensate for lower absolute radiation doses (25, 26), we decided to assess whether this methodology will lead to improved therapeutic results. We chose, for this purpose, a metastatic human colonic carcinoma model that represents a relatively radioresistant tumor type (1).

We compared the therapeutic efficacy of standard chemotherapy with 5-fluorouracil/folinic acid (9–12) to radioimmunotherapy with 131I-labeled anti-CEA antibodies. CEA, which is a Mr 180,000 glycoprotein anchored in the cell membrane via a glycosyl-phosphatidyl-inositol moiety, was described by Gold and Freedman (27) 30 years ago as one of the first tumor-associated antigens. Its excellent suitability as a target antigen for radiolabeled antibodies has been demonstrated experimentally as well as clinically (28).

MATERIALS AND METHODS

Animal Model. GW-39 human colon cancer intrapulmonary metastases were induced by i.v. injection of 30 μl of a 10% suspension of GW-39 tumor (29) in nude mice, as has been described previously (20, 30). Multiple (as many as 100) microscopic tumor colonies develop in the lungs of such animals, reaching a size of approximately 1–3 mm at 4 weeks after tumor cell inoculation (Fig. 1). With high reproducibility, the animals begin to lose weight by 3–6 weeks and eventually die at 5–8 weeks after tumor inoculation.

Radioiodination. Purified MN-14 anti-CEA murine monoclonal antibody, a new generation, high-affinity anti-CEA antibody (Kd = 109 l/mol; supplied by Immunomedics, Inc., Morris Plains, NJ) has been used in experimental and clinical radioimmunotherapy trials (31, 32). LL2 anti-CD22 B-cell lymphoma monoclonal antibody was used as the nonspecific antibody. F(ab)2 fragments were generated with pepsin and then purified by passage over a protein A column. The unbound fraction was ultrafiltered exhaustively (YM-30 membrane; Amicon, Danvers, MA) and finally dialyzed against PBS (0.04 M phosphate, 0.15 M NaCl, and 0.02% NaN3), pH 7.4. The Fab was prepared from the F(ab)2 by reduction with cysteine. The purity of each agent was evaluated by SDS-PAGE using reducing and nonreducing conditions, size-exclusion HPLC, and immunoelectrophoresis. All antibodies were radioiodinated by the chloramine-T method (33). Protein-bound iodine was separated from free iodine by passage over a PD-10 column (Pharmacia, Piscataway, NJ) equilibrated with 0.04 M phosphate, 0.15 M NaCl, and 0.02% NaN3, pH 7.4. The Fab was prepared from the Fab by reduction with cysteine. The purity of each agent was evaluated by SDS-PAGE using reducing and nonreducing conditions, size-exclusion HPLC, and immunoelectrophoresis. All antibodies were radioiodinated by the chloramine-T method (33). Protein-bound iodine was separated from free iodine by passage over a PD-10 column (Pharmacia, Piscataway, NJ) equilibrated with 0.04 M phosphate, 0.15 M NaCl, and 0.02% NaN3, pH 7.4, containing 1% human serum albumin. Specific activity of the labeled product was 12–15 μCi/μg of IgG. Routine quality assurance of radioiodinated antibody showed no detectable aggregates and 2–4% free radioiodine by size exclusion HPLC using a Zorbax GF-250 (DuPont, Wilmington, DE) column. The MTD of radioantibody (250 μCi) was administered by i.p. injection in 0.1–0.25 ml of buffer, and the dose delivered was monitored with a Deluxe Isotope Calibrator II (Nuclear Associates).

Biodistribution Studies. Nude mice bearing 3.5-week-old GW-39 intrapulmonary micrometastases were injected i.v. with either 10–20 μCi (1.0–2.0 μg) of 131I-labeled IgG or F(ab)2 or Fab. Groups of four to five animals per time interval were given sodium pentobarbital, bled by cardiac puncture, and then killed by cervical dislocation. Time intervals for 131I-labeled...
labeled IgG were 1, 3, 7, and 14 days; for 131 I-labeled F(ab)_2 were 6 h and 1, 3, and 7 days; and for 131 I-labeled Fab were 2 and 6 h and 1 and 2 days. Lung nodules were excised along with other organs, weighed, and counted in a gamma scintillation counter using appropriate windows for each isotope.

**Dosimetry.** Radiation doses and dose rates to 3.5-week-old tumor nodules were calculated for monovalent Fab versus bivalent F(ab)_2 and IgG on the basis of the biodistribution data, the respective activities administered for therapy [260 μCi 131 I-labeled IgG versus 1200 μCi F(ab)_2 versus 3.0 mCi Fab], and the absorbed fractions for peripheral (IgG) versus homogeneous (Fab) distribution in a tumor with a 0.5-mm radius. Radiation doses to tumor nodules were calculated by assuming a spherical geometry. Cumulated activities were derived by integrating the biodistribution data over time, and doses were calculated based on self-to-self doses in spheres by assuming absorbed dose fractions as described by Siegel and Stabin (34) and others (35, 36). A strictly peripheral accumulation was assumed for IgG, in contrast to a completely homogeneous distribution for Fab fragments. For bivalent F(ab)_2 fragments, the arithmetic mean of absorbed fractions for peripheral and homogeneous distribution was used.

**Microautoradiography.** Mice implanted with tumor and given 131 I-labeled antibody were sacrificed at defined time intervals. The tumor was removed, stored in 10% formalin, and paraffin-embedded, and 5-μm sections were mounted onto glass slides. The sections were heat-fixed, deparaffinized with xylene and graded alcohol, and coated with NTB3 emulsion (Eastman Kodak, Rochester, NY) and stored at 4°C. After 2 weeks, the slides were developed using a 2-min incubation in 1:1 Dektol at 15°C, followed by a 5-min incubation in Kodak Rapid-Fix. After a 30-min wash with continuous flow of fresh water, the slides were counterstained with H&E.

**Therapy Studies.** Treatment was initiated on day 1, 3, 7, or 14 days after tumor cell inoculation. Animals either received chemotherapy with 5-fluorouracil/folinic acid or were given equitoxic radioimmunotherapy. For radioimmunotherapy, animals received injections of a single dose of 131 I-labeled MN-14 Fab, F(ab)_2, or IgG, each at its respective MTD. The MTDs of these radioiodinated antibody fragments had been determined as described in detail earlier (26). In a s.c. GW-39 human colon cancer model, 10–15% dose escalations were performed. The MTD was defined as the highest possible dose under the respective conditions that did not result in any animal deaths, with the next higher dose level resulting in at least 10% of the animals dying. MTD levels obtained in this s.c. model were applied for therapy in the lung metastasis model described in the present manuscript. Twenty animals were studied in each treatment group. As a nonspecific therapy control, the 131 I-labeled LL2 (formerly referred to as EPB-2) was used (37), which recognizes an antigenic determinant (CD22) not found on GW-39 colon carcinomas. Body weight was recorded weekly, and survival was monitored. The MTD was defined as the highest possible dose under the respective conditions that did not result in any treatment-related death (26). Animals were observed until their death, or if they survived for >30 weeks, they were removed from the group and sacrificed for histopathological examination. For chemotherapy, the mice received an i.v. injection of 1.8 mg leucovorin, followed by 0.6 mg of 5-fluorouracil 1 h later, on 5 consecutive days, each in 200 μl of saline to mimic the clinically typical standard chemotherapeutic regimen given colorectal cancer patients (38). This is the MTD in mice, whereas higher doses led to dose-limiting mucositis and diarrhea.

**Bone Marrow Transplantation.** Bone marrow was harvested using sterile technique from untreated donor BALB/c mice. Total cells were counted by hemocytometer. Cells were diluted, and 1 × 10^7 cells were injected i.v. into recipient mice 6–8 days after radioantibody administration in 100 μl of buffer. The number of cells and the time for BMT have already been established (39).
RESULTS

For all three $^{131}$I-labeled immunoconjugates [IgG, F(ab)$_2$, and Fab], the red marrow was the only dose-limiting organ; maximum tolerated activities were 260 µCi for IgG, 1.2 mCi for F(ab)$_2$, and 3.0 mCi for Fab. Bone marrow transplantation (42, 43) was able to increase this MTD by 30% (IgG) to 60% (Fab). In contrast to our earlier observations with radiometal-conjugated fragments (25), no sign of radiation nephrotoxicity was found with radioiodinated ($^{131}$I) fragments, which is in accordance with radiation doses of <10 Gy to the kidneys (34). However, there was a drop in body weight (~20%) at weeks 1–2 after therapy.

Untreated animals died from rapidly progressing pulmonary metastases within 4–8 weeks after tumor inoculation. Histologically, the lung parenchyma shows increasing amounts of tumor involvement with time after tumor cell transplantation (Fig. 1, A–C) and was almost completely replaced by tumor at the time of death (Fig. 1D). The irrelevant radiolabeled IgG prolonged life for only 2–4 weeks (Fig. 2) when given 3 days after tumor inoculation, and 5-FU/leucovorin chemotherapy led to a mean prolongation of survival of 6–7 weeks. The tumor-specific radiolabeled antibodies performed significantly ($P < 0.001$) better in all cases (Figs. 2–4). At equitoxic dosing (i.e., at their respective MTD), all three $^{131}$I-labeled MN-14 immunoconjugates [IgG, F(ab)$_2$, and Fab] led to a ~90% cure rate when given 3 days after tumor inoculation (Fig. 2). One hundred % of tumors were cured when animals were dosed 24 h after i.v. tumor cell injection (data not shown). In 1-week-old tumors, chemotherapy led to a mean life prolongation of only 4 weeks, whereas radioimmunotherapy still led to a 55–80% cure rate, depending upon the immunoconjugate chosen ($P < 0.001$). The estimated probability of survival, $Pr(T > t)$ ± SE, at 30 weeks was 80.0 ± 8.9% for the Fab and 55.0 ± 11.1% for the IgG. In 2-week-old tumors, $^{131}$I-labeled IgG was virtually unable to achieve cures (only 1 of 20 animals), whereas $^{131}$I-labeled Fab was still successful in curing 35% of tumors, again also being superior to bivalent fragments ($t = 7.246; P < 0.01$). Therapeutic effects could also be appreciated macroscopically as well as histologically when mice were treated with either the $^{131}$I-labeled IgG (Fig. 3B) or the $^{131}$I-labeled Fab (Fig. 3C). Tumor colonies were necrotic 3 weeks after a therapeutic dose of $^{131}$I-labeled Fab (Fig. 4A), and no evidence of tumor existed at 30 weeks after therapy. No necrotic cavities could be detected in any of the surviving mice. Normal lung parenchyma seems to regenerate (Fig. 4B). In contrast, regions of tumor regrowth were observed at the same time after $^{131}$I-labeled IgG therapy (Fig. 4C).

To analyze the radiation dosimetry in the present model, well-counter biodistribution studies were performed with the respective $^{125}$I-labeled immunoconjugates in mice bearing 3.5-week-old pulmonary tumors (i.e., approximately 1–3 mm in size). The biodistribution studies of $^{131}$I-labeled MN-14 [as complete IgG, F(ab)$_2$, and Fab] in 3.5-week-old pulmonary metastases show that, despite higher absolute uptake values with IgG than with fragments, the therapeutic ratios, in terms of tumor: blood ratios, were highest with monovalent Fab (Fig. 5). Additionally, microautoradiography showed that IgG displays a strong accumulation in the tumor periphery, whereas Fab fragments reveal the most homogeneous distribution within the tumor (Fig. 6). Interestingly, dosimetric estimates based on these assumptions gave very similar results for absolute tumor doses with IgG, bivalent fragments, and monovalent Fab (14.4 Gy for $^{131}$I-labeled IgG as compared with 17.1 Gy for F(ab)$_2$ and 16.7 Gy with Fab). However, large differences were found with respect to intratumoral dose rates. Because of their rapid and homogeneous uptake as well as high maximum tolerated activities, monovalent Fab fragments displayed 5–7-fold higher initial and maximum dose rates as compared with complete IgG (Fig. 7).

DISCUSSION

The excellent therapeutic performance of $^{131}$I-labeled immunoconjugates observed in this micrometastatic model is interesting from the dosimetric perspective, because it has been argued that $^{131}$I may not be suitable for treating micrometastatic disease (35, 36). Given a mean path length of the $\beta$ particles of $^{131}$I in tissue (~0.5 mm), it has been hypothesized that $^{131}$I may not be optimal for treating micrometastases, because the loss of
A significant proportion of the decay energy outside of the tumor might be expected and, thus, this lost energy would not contribute to the therapeutic effect.

This more advanced tumor stage was chosen for biodistribution studies, because this stage would allow the isolation of tumor nodules from surrounding lung tissue with sufficient reliability for uptake quantitation to be measured in the scintillation counter. However, because, as we have shown earlier for macroscopic tumors (44, 45), we found evidence for an exponentially increasing tumor uptake with decreasing tumor size at the microscopic level as well, the dosimetric considerations based on these larger tumors may well underestimate the actual tumor uptake values occurring when treating earlier stages having smaller tumor nodules. On the other hand, decreasing absorbed fractions with decreasing tumor size (Fig. 5A) would counteract the tendency toward higher radiation doses with

---

**Fig. 4** The microscopic effects of therapy on colon cancer metastases in the lungs. A, mostly necrotic areas in the lung metastases at 3 weeks after injection of 131I-labeled MN-14 Fab into 14-day-old inoculated animals. B, no evidence of tumor in animals surviving 30 weeks. C, tumor regrowth from surviving malignant clones 30 weeks after treatment with 131I-labeled MN-14 IgG (100-fold magnification; H&E stain).

---

**Fig. 5** Pharmacokinetic and dosimetric considerations of the observed biological effects. A, intratumoral absorbed beta doses of 131I in relation to the tumor radius for peripheral (IgG) and homogeneous (Fab) radionuclide distribution. B–D, comparative biodistribution kinetics in the tumor and the blood with 131I-labeled IgG (upper right panel), F(ab)2 (lower left panel), and Fab (lower right panel).
decreasing tumor size. Although the absolute dosimetric numbers discussed in the following paragraphs may, therefore, deviate from those occurring in the real therapeutic setting, there are, nevertheless, trends that may help explain the observed biological effects.

Differences in intratumoral distribution of IgG and Fab fragments is a well-known phenomenon, attributable to a variety of physicochemical factors in the tumor (16, 17). On the other hand, a strictly peripheral accumulation will lead to the loss of almost half of the β energy outside the tumor, as compared with a strictly homogeneous intratumoral distribution (35, 36).

Higher therapeutic efficacy of identical doses, when given at higher dose rates, is a well known phenomenon in external beam radiation (46, 47) but has not been investigated yet in sufficient detail for internal emitters (26). These data strongly support the hypothesis that dose rate effects may be crucial, not only at the comparably high levels usually studied with external beam radiation but also in the ranges that are lower by several orders of magnitude than with internal emitters.

The fact that the maximum tolerated doses of complete IgG, bivalent or monovalent fragments have been reached at grossly different activities is attributable to the marked differences in clearance of the three conjugates (the lower the molecular weight, the faster the clearance; thus, the lower the red marrow dose/unit injected activity). Most likely because of differences in dose rate (48), this results in red marrow doses of ~16 Gy for 131I-labeled IgG, 8 Gy for 131I-labeled bivalent fragments, and 4 Gy for monovalent fragments at their respective maximum tolerated activities. Because, on the other hand, dosimetric estimates gave very similar results for absolute tumor doses with IgG, bivalent fragments and monovalent Fab (14.4 Gy for 131I-labeled IgG as compared with 17.1 Gy for F(ab)2 and 16.7 Gy with Fab), differences in therapeutic efficacy cannot simply be a reflection of differences in dosing.

In summary, these results suggest that in an adjuvant therapy or minimal residual disease setting, targeted radio-nuclide therapy is superior to conventional chemotherapy. In contrast to current dogma, smaller (e.g., monovalent) fragments are therapeutically superior to bivalent antibody immunoconjugates, most likely because of a more homogeneous tumor uptake and penetration, as well as higher initial dose rates. Indeed, higher therapeutic ratios in terms of tumor: blood ratios, a more homogeneous tumor uptake and penetration, and higher initial dose rates may compensate for the loss of affinity and lower absolute uptake values characteristic of monovalent fragments. This encourages the development of even smaller, receptor-binding peptides for therapeutic purposes. Even if the renal accretion of such molecules may become critical, radiation nephrotoxicity can be prevented reliably, when used in conjunction with D-lysine (24, 25). These findings encourage clinical studies with radio-labeled monovalent antibody fragments or even smaller molecular recognition units (e.g., peptides) in patients with minimal residual disease or in the postsurgical adjuvant therapy setting. In conclusion, radioimmunotherapy, particularly with antibody Fab fragments, may represent a new strategy...
for treating disseminated cancer cells, which are considered to be the basis of metastasis and the principal cause of cancer mortality.

REFERENCES


Cure of Metastatic Human Colonic Cancer in Mice with Radiolabeled Monoclonal Antibody Fragments

Thomas M. Behr, Rosalyn D. Blumenthal, Stavros Memtsoudis, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/6/12/4900

Cited articles
This article cites 41 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/6/12/4900.full#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/6/12/4900.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.