Local Distribution and Concentration of Intravenously Injected $^{131}$I-9.2.27
Monoclonal Antibody in Human Malignant Melanoma

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ABSTRACT

Regional measurements of $^{131}$I-9.2.27 distribution in human melanoma tumors were obtained using quantitative autoradiography. Tumors were removed from patients 72–96 h after they had received an i.v. injection of 9.15 mCi (100 mg) of $^{131}$I-9.2.27. The autoradiographic images showed that the radioactivity reaching the tumor was heterogeneously distributed. Areas of relative high and low uptake were selected in each tumor. Regions of high activity contained from 51 to 1371 nCi/g, while areas with low uptake had radioactivity ranging from 12 to 487 nCi/g. The reliability of the autoradiographic measurements was demonstrated by the strong positive correlation with direct tissue sample counting (r = 0.994, P < 0.001). Since comparative immunocytochemistry showed a homogeneous and diffuse staining of target antigen on viable tumor cells, variability of monoclonal antibody uptake within individual tumors was not primarily due to heterogeneity of antigen expression in these cases. However, antigen levels accounted for some of the variation from tumor to tumor. When immunoperoxidase staining was repeated on adjacent sections without the addition of 9.2.27, it confirmed the nonuniform distribution of monoclonal antibody found at autoradiography. Thus, quantitative autoradiography provides information about the distribution and local concentration of radioactive antibody in tumors allowing calculation of the radiation dose delivered to small regions within tumors.

INTRODUCTION

Recent therapeutic studies with radiolabeled MoAbs have reported objective, in some cases dramatic, but transient tumor responses (1–3). Many factors may alter a solid tumor response to radioimmunotherapy (4–5), but two of the most important are the distribution and local concentration of activity within the tumor. The purpose of the present study was to determine the actual amount of radioactivity reaching the tumor and to evaluate the pattern of radioactivity within the tumor by means of quantitative autoradiography. We used monoclonal antibody 9.2.27, which recognizes the M, 250,000 proteoglycan (p250) that is present on the cell surface of a high percentage of human melanoma tumors (6–7). MoAb 9.2.27 selectively targets on the melanoma cell surface after intravenous administration (8) and can be specifically radiocytotoxic in vitro, when labeled with iodine-125 (9).

MATERIALS AND METHODS

Antibody and Radioiodination. The 9.2.27 murine antimelanoma monoclonal antibody (IgG2a) was radioiodinated by the chloramine T method (10). For each milligram of 9.2.27 we used 8.6 mCi of $^{131}$I label (New England Nuclear, North Billerica, MA) and 23 µg of chloramine T (Malinckrodt, Inc., Paris, KY). The reaction was quenched after 5 min by the addition of 80.5 µg sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ). The radiolabeled protein was separated from free iodine by Sephadex G10 gel chromatography (Pharmacia, Uppsala, Sweden). The final product had a specific activity of 6.9 mCi/mg and contained less than 3% of free iodine as assessed by instant thin-layer chromatography. Each $^{131}$I-9.2.27 radiopharmaceutical product was tested for endotoxin and sterility. The concentration of the antibody was adjusted by the addition of unlabeled 9.2.27, so that each patient received 100 µg of 9.2.27. The mean administered activity was 9.15 mCi of the $^{131}$I-labeled antibody.

Radioantibody Quality Control. The immunoreactivity of the radiolabeled monoclonal antibody was assessed by a cell binding assay. Briefly, p250 positive human melanoma cells (FEMX), in concentrations ranging from 1 to 8 million cells in 0.2 ml, were incubated with 5 µg of radiolabeled 9.2.27 for 1 h at 22°C. An excess (25 µg) of unlabeled 9.2.27 was used to assess the nonspecific binding. The cells were washed in phosphate buffered saline 1% bovine serum albumin and separated and centrifuged. The supernatant was aspirated and the cell pellet was counted in a gamma counter (United Technologies-Packard, Downers Grove, IL). After subtracting the nonspecific binding, the immunoreactive fraction (expressed as a percentage) was calculated as the ratio of the maximal radioactivity specifically bound to cells divided by the total activity added (11). The mean value of the immunoreactive fraction was 43% (34–47%). Trichloroacetic acid (Baker Co., Phillipsburg, NJ) precipitation and size-exclusion high-performance liquid chromatography were also used to assess the percentage of protein bound iodine-131 and the chemical purity of the radiolabeled compound.

Patients. Six patients with histologically confirmed metastatic malignant melanoma were studied under a National Cancer Institute approved protocol. Before entering the study, patients with multiple lesions underwent a preliminary biopsy. Tumor nodules were evaluated for antigen expression by immunoperoxidase staining. Patients received 9.15 mCi of $^{131}$I-9.2.27 (100 mg) through 2 h of i.v. infusion. Serial blood samples were obtained at 5 and 30 min, and at 1, 2, 4, 24, 48, 72, and 96 h after infusion for determination of plasma clearance. The percentage of the injected dose present in the vascular compartment was determined using plasma and blood volume estimates derived from a nomogram (12). Precipitation in trichloroacetic acid was performed on serum samples to determine the fraction of protein bound $^{131}$I. Patients underwent surgery 72–96 h after monoclonal antibody administration. Subcutaneous tumor nodules with a diameter ranging from 0.5 to 1.1 cm were removed and processed for tissue counting, histology, immunoperoxidase staining, and quantitative autoradiography. For each patient, the same tissue block was used for all procedures. Sections used for autoradiography were usually adjacent to those for immunoperoxidase staining. The only exception was Patient 5, whose tumor was counted and then divided into two blocks, one for immunohistochemistry and one for autoradiography.

Tissue Counting and Immunoperoxidase Staining. Excised s.c. tumor nodules were weighed and assayed for radioactivity in a gamma counter. Radioactivity, expressed as cpm/g of tissue, was compared to a standard of the patient dose. The percentage of injected dose per gram of tissue (%ID/g) was calculated. After counting, the tumor blocks were processed for immunoperoxidase staining and for conventional histology. The avidin-biotin method (13) was used and staining with diaminobenzidine substrate was scored from negative to four plus (+ + + +). In order to
assess the in vivo localization of 9.2.27 after the i.v. injection, the immunoperoxidase staining was repeated on adjacent sections without adding 9.2.27 as the primary antibody.

Quantitative Autoradiography. The general features of quantitative autoradiography have recently been reviewed (14, 15). Frozen sections of 20-μm were cut at −20°C in a cryomicrotome corresponding to the largest cross-sectional area of the tumor. Sections were dried by heating at 65°C and placed in a light-tight cassette along with 131I-calibrated standards and X-ray film (SB5-Kodak). Sections (20 μm) adjacent to those for autoradiography were also cut for histological correlation. These sections were fixed in 10% buffered formalin (Baker Chemical Co., Phillipsburg, NJ) and stained with hematoxylin & eosin. Iodine-131-calibrated standards were prepared with a modification of a previously described technique (16). Briefly, serial dilutions of Na131I were prepared ranging from 15 nCi/ml to 15,000 nCi/ml. Two hundred mg of gelatin (Sigma Chemical Co., St. Louis, MO) were then added to 0.8 ml of each dilution in polyprene tubes that were capped and heated to 50°C for 5 min, until the gelatin melted. After gently mixing the suspensions, 200-μl aliquots were weighed and counted in a gamma counter to assess the radioactivity per gram. The remaining part of each suspension was then frozen in precooled liquid dichlorodifluoromethane (VWR Scientific Inc., CA) at −40°C as frozen gelatin sticks. The sticks were mounted on cryostat object holders, 20-μm sections were cut at −20°C and air dried at 22°C. After 2 weeks of exposure, the autoradiogram films were photographically processed. The autoradiographic images were digitized using a computerized scanning microdensitometer (P-1000 HS, Optronics International Inc., Chelmsford, MA). Optical density measurements within 50 × 50 μm elements of the autoradiographic images were obtained and stored by an on line computer (PDP 11/60; Digital Equipment Corp., Maynard, MA) equipped with two video monitors. The optical density measurements over the autoradiographed iodine-131 standards were plotted against their respective level of radioactivity and a standard curve was generated by performing a polynomial fit of the data (14). Regions of interest were drawn over selected tissue areas and regional optical density measurements were converted to nCi/g of tissue by means of the standard curve. The selection of autoradiographic areas was facilitated by projecting the images of adjacent H & E histological sections on the second monitor by means of a video camera system (Sierra Scientific Corp., Mountain View, CA). A computerized aligning procedure was used to superimpose the digitized histological images to the correspondent autoradiographic images. A large region of interest was drawn over the entire area of the tumor so that the mean radioactivity content per gram of tumor was obtained. Two smaller areas were drawn around those tumor regions with the highest and the lowest optical density measurements. These corresponded to the highest and lowest levels of radioactivity in the tumor, respectively. The autoradiograms were compared to the adjacent histological sections so that activity levels could be related to histological structures. The same analysis was performed on available normal tissue. The final autoradiographic results were decay corrected so that the values obtained reflected the activity at the time of surgery.

RESULTS

Table 1 summarizes patient and antibody data. Plasma clearance curves showed that 86% of the injected dose was present in the vascular compartment at the end of infusion. At the time of biopsy 8–17% of the injected dose was retained in the plasma, with a mean precipitable fraction of 96%. The mean value of t½ beta for plasma clearance was 38 h (Table 1).

The comparative findings of histology, immunoperoxidase staining, and autoradiography are shown in Table 2. Histologically, the tumors were circumscribed or infiltrative in configuration. In either form, epithelioid cells were found growing in noncontiguous nests or in a packed pattern interspersed with the stroma. Scattered lymphocytes were also often observed. Pigment was present in the cytoplasm of tumor cells or deposited in areas of tumor necrosis. The tumor of Patient 4 showed abundant pigmentation and a large central necrotic area.

Immunoperoxidase staining, performed with the addition of 9.2.27, showed a homogeneous and diffuse staining of target antigen on viable tumor cells (Figs. 1c and 2c). Minimal background staining was present in areas of stroma. The highest antigen content was obtained in tumors of Patients 1 and 2. Because of the abundant melanin present, it was impossible to grade the tumor of Patient 4.

The autoradiographic images showed the distribution of radioactivity within the tumor. Macroscopically, these matched

Table 2 Comparative findings in melanoma tumors from patients who received an i.v. injection of131I-9.2.27

<table>
<thead>
<tr>
<th>Patients</th>
<th>Tumor</th>
<th>Microscopic</th>
<th>Immunoperoxidase (in vitro)</th>
<th>Autoradiography pattern</th>
<th>Immunoperoxidase (in vivo)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Grade</td>
<td>Pattern</td>
<td>Grade</td>
</tr>
<tr>
<td>1. CC</td>
<td>Circ</td>
<td>Viable tumor cells</td>
<td>4+</td>
<td>Homogeneous</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td>2. BB</td>
<td>Circ</td>
<td>Viable tumor cells</td>
<td>3+/4+</td>
<td>Homogeneous</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td>3. BF 1</td>
<td>Infill</td>
<td>Viable tumor cells</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>4. CG</td>
<td>Circ</td>
<td>Viable tumor cells</td>
<td>No</td>
<td>Focal, high intensity</td>
<td>1+</td>
</tr>
<tr>
<td>5. SM</td>
<td>Circ</td>
<td>Viable tumor with central necrosis</td>
<td>2+/3+</td>
<td>Homogeneous</td>
<td>0+</td>
</tr>
<tr>
<td>6. MC</td>
<td>Circ</td>
<td>Viable tumor cells</td>
<td>3+</td>
<td>Homogeneous</td>
<td>Heterogeneous</td>
</tr>
</tbody>
</table>

Abbreviations: Pathology: circ, circumscribed discrete tumor nodule; infil, infiltrating tumor. Immunoperoxidase (in vitro), immunoperoxidase staining performed with the addition of 9.2.27 as primary antibody. Immunoperoxidase (in vivo), immunoperoxidase staining performed without added primary antibody. Autoradiography: In general the limits of the activity in autoradiographic images matched the histological limits of the tumor. Stroma had low activity. ND, not done; no grade, grading not possible for heavily pigmented tumor.

Grade 0, background activity.

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Grade 0, background activity.
Fig. 1. a, histological section of melanoma tumor from Patient 1 for comparison with Fig. 1b. Arrows, tumor border (H & E staining). b, autoradiogram obtained from an adjacent section of the same tumor showing the distribution of $^{131}$I-9.2.27. Note the circumscribed activity that matched the corresponding histological profile of the tumor. Note also the diffuse and heterogeneous distribution of radioactivity within the tumor. The values for local tracer concentration are shown in the gray scale on the right. c, immunoperoxidase staining performed on a section of the same tumor with the addition of 9.2.27 as primary antibody. Note the diffuse and homogeneous staining of tumor cells (x 120). d, immunoperoxidase staining performed on an adjacent section of the same tumor without the addition of 9.2.27 as primary antibody. Note the diffuse but heterogeneous staining of tumor cells (Counterstaining with Hematoxylin x 120).

Table 3 shows the results of the quantitative autoradiographic measurements on these excised tumors expressed as mean ± SD. High activity and low activity regions were identified as described above. In each specimen there was a large variation of the radioactive content from region to region. Within a tumor, areas of high uptake could contain from two to 17 times more radioactivity than regions with low activity. Autoradiographic measurements obtained over the whole tumor area were indicative of the mean radioactivity content and reflected the relative abundance of the high and low activity regions in the same tumor.

Table 4 shows the autoradiographic measurements of normal tissues obtained from three patients. The tissues included skin, subcutaneous fat, and stroma that were adjacent to the tumor biopsy. The variation of radioactivity in these normal tissues was not as great as that found in the tumor. Furthermore, measurements on the regions of highest uptake were less than those found in the tumor. The highest tumor to normal tissue ratio was observed in Patient 1 (Fig. 1b), whose tumor contained seven times more radioactivity than that found in the surrounding normal tissue. This ratio was 10:1 when areas of highest tumor activity were used in the calculation.

We compared the results of gamma well counting of lesions expressed as cpm/g and %ID/g with the values obtained for the...
Fig. 2. a, histological section of a melanoma tumor from Patient 2 for comparison with Fig. 2b (H & E staining). b, autoradiogram obtained from an adjacent section of the same tumor showing the distribution of $^{131}$I-9.2.27. Note the focal high activity region in the center that was associated to viable tumor in proximity of a vascular structure. Other similar vascular structures were found throughout the adjacent histological section with no correspondent focal activity. c, immunoperoxidase staining performed on a section of the same tumor with the addition of 9.2.27 as primary antibody. Note the diffuse and homogeneous staining of tumor cells (X 31). d, immunoperoxidase staining of an adjacent section of the same tumor performed without the addition of 9.2.27 as primary antibody. Arrow, a cluster of positive cells in proximity of a vascular structure (counterstaining with hematoxylin; X 31).

whole tissue section using quantitative autoradiography (Table 5). For Patients 1 and 3, measurements over the whole section differed from the corresponding values reported for the whole tumor because the sections included some normal tissue. The gamma counter measurements of the tissue blocks were linearly related to the autoradiographic measurements with a strong positive correlation ($r = 0.994$, $P < 0.001$).

DISCUSSION

The present study reports the findings in seven metastatic s.c. melanoma tumors removed from patients after they had received i.v. doses of $^{131}$I-labeled antimelanoma antibody. The tumors were evaluated for histology by hematoxylin & eosin staining, antigen expression by immunocytochemistry, regional distribution of radioactivity by quantitative autoradiography, and overall radiotracer uptake in tissue by gamma well counting. Two general conclusions could be drawn from our results. First, there was great variability in the uptake of this radiolabeled MoAb by different tumors. Second, within individual tumors the distribution of activity was not uniform. Evaluation of small regions of tumor sections using quantitative autoradiography showed that within a single tumor, activity could vary by as much as 17-fold.

The variability in tumor uptake of injected radiolabeled monoclonal antibody could account for the differences in sensitivity reported by many authors in clinical studies with MoAbs (17–21). Factors that affect tumor uptake include the dose of antibody administered, the serum levels of antibody, antibody immunoreactivity, tumor size, and antigen content. Patients in this study received the same dose of MoAb. Immunoreactivities of the several moAb preparations were about the same. The plasma clearance curves did not show significant differences among patients and the size of the tumors studied were about the same. These factors, therefore, did not significantly affect the variation among patients in the present study. On the other hand, there was general agreement between the grade of immunoperoxidase staining, performed with the addition of 9.2.27, and the autoradiographic results. The two patients with the highest uptakes had 4+ and 3+/4+ tissue antigen levels, respectively. Lower uptakes were found in tumors with 2+ and 3' antigen levels. This suggests that target antigen concentration was a major factor that caused differences of MoAb uptake among the different tumors in this study. Additional important
factors to be considered include tumor cellularity, capillary permeability, and local blood flow.

The autoradiographic images and the histological sections were superimposable so that areas of high and low radioactivity could be related to specific regions within the tissue section. Areas of high uptake were always associated with viable tumor, but the presence of a large number of tumor cells did not guarantee that uptake in the region would be high. Regions of very low uptake were most often associated with areas of stroma or tumor necrosis. Since the target antigen was diffusely and homogeneously distributed in viable tumor cells, the local high concentrations were not due to increased antigen density. Nevertheless, p250 is a membrane bound antigen and its absence or negligible amount in regions with low cellularity may cause poor MoAb uptake.

Immunoperoxidase staining of the tissue sections performed without the addition of 9.2.27 as the primary antibody showed that the pattern observed at autoradiography indeed represented antibody uptake and not just radioactivity localization. Although an exact correlation could not always be achieved, the distribution pattern of endogenously bound 9.2.27 was similar to the autoradiographic pattern. Furthermore, the lack of staining in two tumors containing detectable amount of radioactivity suggests that staining for endogenously bound antibody with immunoperoxidase is less sensitive than quantitative autoradiography in assessing in vivo localization. It was of interest to observe that the focal area of intense immunoperoxidase staining in the tumor from Patient 2 was located next to a vascular structure. Other vascular structures were found in the same section but were not associated with focal staining of surrounding tumor cells. One possible interpretation of this observation is related to tumor blood supply. Previous studies have shown that the functioning microvasculature of a tumor may represent only part of the total tumor vascular bed (22-24). Furthermore,
large local variation of tumor blood flow may occur without specific differences between peripheral and central regions (25). An inverse relationship between blood flow and tumor growth has also been found (26).

The digitization of the autoradiographic images allowed us to quantitate activity within small regions of the tumors. In this way, radioactivity maps could be generated for individual tumor sections. We evaluated the midsection of the tumor nodules and found a strong positive correlation between autoradiographic measurements over the whole sections and the activity obtained by counting the whole nodules in a gamma counter. This finding confirmed the validity of the autoradiographic measurements.

Quantitative autoradiography has been extensively used to determine local cerebral blood flow (27), cerebral glucose utilization (28), and capillary permeability (29), as well as regional distribution of radiolabeled compounds within tumors (30). Furthermore, distribution studies of monoclonal and polyclonal antibodies have been performed in animal models (31–33). Quantitative autoradiography also provides data that can be used to calculate the radiation dose delivered to microscopic areas within a tumor. As an example, the uptake in tumor from Patient 1 varied from 0.0063 to 0.0177% of the injected dose per gram. Since the mean biological half-life for 9.2.27 resin is 72 h, the doses delivered to different tumor regions could be calculated. For a patient dose of 100 mCi, this tumor could receive local doses ranging from 600 to 1700 rads. Previous studies with an 111I-labeled anti-p97 Fab fragment found that melanoma tumors received an estimated dose of 1040 rads (1). Applying quantitative autoradiography to study the distribution of radiolabeled antibody in tumors provides important information about the localization of antibody within tumors. It also yields data useful for calculating the radiation dose to small areas in individual tumors. We have shown that tumor uptake of radiolabeled MoAb is not uniform and, therefore, that the dose delivered to tumor cells will not be uniform. Although tissue counting provides helpful information, these data represent the mean radioactive content and do not indicate what portion of the tumor may escape lethal radiation.

In conclusion, we used quantitative autoradiography to measure the local concentration of i.v. injected 111I-9.2.27 in tumors of patients with melanoma. We found that MoAb localization varied from tumor to tumor and also varied locally within individual tumors. Factors that might cause such variation include regional differences in cellularity, permeability, and blood flow. Tumor antigen concentration, while affecting the absolute uptake of antibody, did not seem to account for the intense local areas of antibody activity. While future attention will undoubtedly focus on clarifying the role of tumor blood flow and vascular permeability, studies are also needed to test the effect of MoAb dose, route of delivery and different administration schedules. The finding that antibody may be concentrated in restricted areas within a tumor suggests that therapy with antibody alone or conjugated with toxins or chemotherapeutic agents may only be effective in treating limited areas of tumors. Alternatively, antibody conjugated with selected longer particle range radioactive emitters may affect larger areas, overcoming the problem of restricted intratumor antibody distribution.

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REFERENCES


LOCAL DISTRIBUTION OF MONOCLONAL ANTIBODY


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