Distribution of Radiolabeled Monoclonal Antibody MX35 F(ab')2 in Tissue Samples by Storage Phosphor Screen Image Analysis: Evaluation of Antibody Localization to Micrometastatic Disease in Epithelial Ovarian Cancer

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ABSTRACT

Our objective was to quantify the targeting of the monoclonal antibody (mAb) MX35 F(ab')2 to micrometastatic epithelial ovarian cancer. This mAb detects a Mr 95,000 glycoprotein with homogeneous distribution on 80% of ovarian tumor specimens. Six patients with minimal residual disease from an imaging trial were injected with 2 or 10 mg of 123I- and 125I-labeled mAb MX35 F(ab')2. Biopsied samples were removed at second-look laparotomy 1–5 days post-i.v. or i.p. infusion of antibody. Serial cryostat sections were stained by indirect immunoperoxidase method for antigen distribution and exposed to storage phosphor screens for quantitative autoradiography. Coregistration of tumor histology, antigen expression, and radionuclide distribution demonstrated specific localization in micrometastatic tumor foci (50 μm to 1 mm) found within tissue stroma. The radiolabeled antibody uptake determined by well scintillation counts ranged between 5.2 and 223.5 × 10⁻⁴ percentage of injected dose/g of tumor tissue for 123I. Specific localization of mAb in tumor was determined by tumor:normal tissue (fat) ratios ranging from 0.9:1 to 35.9:1 for 125I. The high resolution and linear response of the storage phosphor imager was used to estimate the radionuclide activity localized in each micrometastatic site. Quantitation of phosphor screen response revealed μCi/g values of 0.026–0.341 for normal tissue and 0.184–6.092 for tumor biopsies, evaluated 4 or 5 days post-antibody injection. The tumor:normal tissue (adjacent to tumor) ratios were between 1 and 4 times greater using the phosphor screen method than well counter measurements, but even larger variations of ratios up to 20:1 were observed between tumor cell foci and stromal cells within the same tissue section. This study has demonstrated that mAb MX35 F(ab')2 localizes to the micrometastatic ovarian carcinoma deposits within the peritoneal cavity. The dosimetry results suggest a therapeutic potential for this antibody in patients with minimal residual disease (<5 mm).

INTRODUCTION

In the treatment of advanced epithelial ovarian cancer, the combined effect of surgery and chemotherapy has resulted in a complete response rate of 45% as confirmed by reassessment surgery (1). However, the risk of recurrence remains high in patients with advanced disease (stages III and IV), with 50% recurring within a median of 14 months after negative second-look laparotomy (2). Patients with residual disease detected at second-look surgery or recurrent disease after completion of initial chemotherapy have a poor prognosis, and few, if any, are cured by currently available salvage therapy. The potential of radiolabeled mAbs4 for the detection of metastatic spread offers significant benefits for the subsequent management of these patients, as well as the possibility to actually treat micrometastatic disease with antibody carrying the appropriate therapeutic radionuclide, toxin, or drug.

The application of radiolabeled antibodies for both radioimmunodiagnosis and treatment of ovarian carcinoma has been ongoing for more than 10 years (3–10). Epenetos et al. (4) and Patiesky et al. (5) used 131I- and 125I-labeled antibodies (HMFG1 and HMFG2) against peptide epitopes of human milk fat globule. Using gamma camera scintigraphy, they successfully demonstrated that >75% of patients having metastatic spread into the peritoneum imaged positively. Negative scans were attributed to the absence of disease or the presence of unresolvable microscopic foci only. The lack of solid tumor nodules >1.5 cm in diameter would render insufficient image

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4 The abbreviations used are: mAb, monoclonal antibody; %ID/g, percentage of injected dose per gram of tissue; cpd, counts per day; T₁/₂, biological half-life; T₁/₂, physical half-life; Tₑ, effective half-life; PET, positron emission tomography.

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contrast to enable specific antibody binding to be detected against a nonspecific background (11). Neither gamma camera imaging nor hand-held surgical radioactivity gamma probes (8, 12) exhibited the sensitivity required to detect micrometastatic disease (<1 cm in diameter) due to insufficient contrast (rarely >10:1) of radionuclide activity accumulation within the tumor relative to the peritumor region (11). Micrometastatic disease may, therefore, remain undetected by conventional nuclear medicine procedures. Moreover, in biodistribution studies using biopsied specimens, the presentation of radioabeled antibody uptake and dosimetry as an activity per unit gram of tissue can be in significant error. This is because of the small size of the biopsy and the presence of only clusters of tumor cells within a large region of stromal tissue, endothelium, and hematopoietic cells. Including nontumor cells in the activity per unit gram calculations can greatly dilute the tumor-specific activity.

To explore ways around this problem, we have examined the use of storage phosphor screen technology to determine the distribution of radioactivity in surgical specimens obtained from an antibody-imaging trial on the use of radioabeled murine mAb MX35 F(ab')2 fragment in patients with ovarian carcinoma having minimal residual disease. Digital images from scanned storage phosphor screens were compared with autoradiographic images obtained using film techniques and MX35 antigen localization determined by indirect immunoperoxidase microscopy in this trial, either paraffin-embedded tumor specimens or fresh, frozen tumor specimens from an earlier surgery were examined by immunohistochemistry for expression of the MX35 antigen on at least 75% of the carcinoma cells. Tissue specimens from six patients who participated in the mAb imaging trial prior to a second-look surgery are summarized in Table 1. Control antibody was not injected into patients for this study.

### Preparation of Radiolabeled mAb MX35 F(ab')2

mAb MX35, a murine IgG1, was generated from the hybridoma fusion of NS-1 murine myeloma cells with spleenocytes from a mouse immunized with four fresh ovarian carcinoma specimens (13) and purified as described previously (14). For fragmentation, purified mAb MX35 IgG (2 mg in 400 μl) was dialysed overnight in 0.1 M citric acid buffer, pH 4.5. Pepsin (25 μg of 1.5 mg/ml; Sigma Chemical Co., St. Louis, MO) was then added to the antibody and digested for 3 h at 37°C with agitation. F(ab')2 fragments were isolated using an Avid Chrom F(ab')2 kit (Uni-Syn Technologies Inc., Tustin, CA). High-yield binding buffer (250 μl) containing 30 μl of antipepsin was added to the antibody-pepsin combination. The entire sample was diluted with 220 μl of high-yield binding buffer and centrifuged to 2000 rpm for 15 min, and the supernatant was placed on a protein A-Avid Chrom cartridge. The unadsorbed fraction was concentrated using a Centricon 30 unit (Amicon, Beverly, MA) at 1075 × g at 4°C. The final antibody concentration obtained was 9.3–13.6 mg/ml. The identity of the fragments was confirmed by SDS-PAGE under reducing conditions; staining of the gel with Coomassie blue revealed bands of Mr 23,000 (light chains) and Mr 107,500 (heavy chains).

The mAb MX35 F(ab')2 fragments were radioabeled with iodine radionuclides using the chloramine-T method as follows: two mg of antibody fragments were added to 0.5 ml of 0.10 M phosphate buffer, pH 7.4. To the radioiodines, 131I and 125I, were added 100 μl of phosphate buffer, and this solution was

### MATERIALS AND METHODS

**Patient Selection.** Patients in this study had undergone prior surgery for epithelial ovarian cancer and had completed a prescribed course of platinum-based chemotherapy. Eligibility criteria included known or suspected carcinoma of the ovary, a Karnofsky performance status greater than 60, no prior administration of murine mAb or fragment, and/or a negative human antimurine antibody titer. Informed consent was obtained from all patients before participation in the study; the study and consent forms were approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center. Prior to participating in this trial, either paraffin-embedded tumor specimens or fresh, frozen tumor specimens from an earlier surgery were examined by immunohistochemistry for expression of the MX35 antigen on at least 75% of the carcinoma cells. Tissue specimens from six patients who participated in the mAb imaging trial prior to a second-look surgery are summarized in Table 1. Control antibody was not injected into patients for this study.

#### Table 1 Summary of 35 biopsied specimens analyzed from six patients with epithelial ovarian cancer

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Number of biopsies from normal tissue</th>
<th>Number of biopsies with tumor cells</th>
<th>Tumor cells per biopsy (%)</th>
<th>Tumor cells immunostained (intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Endometrioid</td>
<td>3</td>
<td>6</td>
<td>&lt;20% clusters</td>
<td>75% (+ to +++)</td>
</tr>
<tr>
<td>2</td>
<td>Serous</td>
<td>3</td>
<td>1</td>
<td>&gt;80% clusters</td>
<td>75% (+ to +++)</td>
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<tr>
<td>3</td>
<td>Endometrioid with clear</td>
<td>2</td>
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<tr>
<td>4</td>
<td>Endometrioid</td>
<td>2</td>
<td>1</td>
<td>&gt;50% clusters</td>
<td>75% (+ to +++)</td>
</tr>
<tr>
<td>5</td>
<td>Carcinoma (poorly differentiated)</td>
<td>6</td>
<td>1</td>
<td>&lt;10% clusters</td>
<td>75% (+ to +++)</td>
</tr>
<tr>
<td>6</td>
<td>Endometrioid (peritoneal)</td>
<td>3</td>
<td>5</td>
<td>&gt;50% clusters</td>
<td>75% (+ to +++)</td>
</tr>
</tbody>
</table>

a Tumor and normal tissue biopsied specimens were examined from all six cases by well scintillation counting and film autoradiography; biopsies from cases 2–6 were analyzed by storage phosphor screen autoradiography.

b The percentage of tumor cells within the stroma of a tissue section was estimated by indirect immunoperoxidase analysis using anticytokeratin antibody to detect carcinoma cells. Cases 1, 4, 5, and 6 showed discrete foci of tumor cells, whereas cases 2 and 3 had tumor cell clusters dispersed throughout the stromal tissue.

c Frozen tissue sections were analyzed by indirect immunoperoxidase analysis using mAb MX35 to evaluate intensity and heterogeneity of antigen expression: +++, strong; + to +++, weak and variable intensity.

To confirm the specific uptake of radiolabeled mAb MX35 F(ab')2, the specific tumor uptake and dosimetry as an activity per unit gram of tissue can be in significant error. This is because of the small size of the biopsy and the presence of only clusters of tumor cells within a large region of stromal tissue, endothelium, and hematopoietic cells. Including nontumor cells in the activity per unit gram calculations can greatly dilute the tumor-specific activity.

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added to the antibody fragment solution. Chloramine-T (2 mg/ml) in phosphate buffer was added, and after 2 min, the reaction was quenched by the addition of 50 μl of sodium metabisulfite (10 mg/ml). The protein was separated by passage through a Biogel P6 column (10 ml; Bio-Rad Laboratories, Melville, NY) using 1% human serum albumin in 0.15 M NaCl as eluant. Terminal sterilization was achieved by filtering through a 0.22 μm filter. Immunoreactivity of the labeled product was determined by sequential absorptions with an antigen-expressing cell line (OVCAR-3). Between 50 and 65% of the radioactivity was adsorbed using the method described by Mattes et al. (14). Percentage of labeled protein was determined by TLC, and incorporation of radiolabeled iodine into protein was >95%. All procedures were performed aseptically with pyrogen-free material.

**Administration of Radionuclidic mAb MX35 F(ab’)_2.** Beginning at least 24 h prior to antibody administration and continuing to the time of surgery, patients were treated p.o. with 10 drops of a saturated solution of potassium iodide three times daily. 125I-labeled mAb MX35 F(ab’)_2 was administered by an i.v. route in a 0.9% sodium chloride solution containing 5% human serum albumin (total volume, 100 ml) through a 0.2 μm Millex G-V filter (Millipore, Bedford, MA) over a period of 1 h. Radiolabeled mAb MX35 F(ab’)_2 was administered by an i.p. route as follows: 500 ml of 0.9% sodium chloride were delivered using a catheter or an existing i.p. port into the peritoneal cavity to facilitate antibody distribution, 100 ml of radiolabeled antibody were added in the same solution as the i.v. route, and an additional 500 ml of 0.9% sodium chloride were delivered. Five patients were entered at the 2-mg antibody dose labeled with both 131I and 125I. Three patients were injected by an i.v. route and two patients by an i.p. route. One patient was entered at the 10-mg antibody dose [2 mg of radiolabeled antibody plus 8 mg of unlabeled mAb MX35 F(ab’)_2] and injected by an i.v. route.

**Blood Samples and Tissue Biopsies.** Blood was drawn prior to radiolabeled mAb infusion, 1–4 h after infusion, during surgery, and 4–7 days after surgery. Whole blood was centrifuged at <2000 rpm for 10 min, and serum was aspirated and stored at −20°C. One ml of “surgery” serum was weighed and then counted in a Packard Cobra well scintillation counter (Packard Instrument Co., Donuers Grove, IL) to compare radiolabeled antibody levels in the blood with those in the biopsied material.

Multiple biopsied specimens, including adjacent normal tissue (fat, muscle, and peritoneal wall) were retrieved from six patients during second-look surgery as summarized in Table 1. Fresh surgical biopsies were divided as follows: one-half of each specimen was paraffin embedded and used for routine histological evaluation in our Department of Pathology. The other half of each biopsy was weighed and counted in a Packard Cobra well scintillation counter and then snap frozen in liquid nitrogen, embedded in OCT compound (Miles Laboratory Inc., Elkhart, IN), and stored at −80°C. A proportion of the frozen surgical biopsies from each case were cut using a motorized cryostat (Bright Instrument Co., Huntingdon, England) and air dried onto microscope slides. Adjacent tissue sections (6-μm thickness) from each biopsy were then analyzed for MX35 antigen localization using an indirect immunoperoxidase procedure and for radionuclide distribution by autoradiography using film and storage phosphor screens.

The number of cpm was obtained in two windows centered at 25 keV for 125I and 364 keV for 131I. The cpm was converted into activity by measuring 125I and 131I standards alongside the tissue specimens. The %ID/g for each radionuclide was determined for the serum and each tissue biopsy by dividing the specific activity (i.e., μCi/g) by the activity administered to the patient and multiplying by 100.

**Immunohistochemical Analysis and MX35 Antibody Localization.** Frozen tissue sections were fixed with cold acetone and analyzed for reactivity with mAb MX35 as described previously (15). Antibody staining patterns were scored in a semiquantitative fashion. Specimens were classified as showing strong (++++) antigen expression when 75% or more of the tumor cells were stained; heterogeneous (+ to ++) expression with variable intensity when 10–75% of the tumor cells stained; and no expression when negative or less than 10% of the tumor stained.

In a xenograft murine model, nonspecific, control antibody L6 was injected for comparison with mAb MX35, both intact and F(ab’)_2. In the animal studies, mAb MX35 (and not mAb L6) targeted specifically to MX35-positive OVCAR-3 human ovarian cancer cells, growing as tumors in the mice. In this clinical trial, a nonspecific, control antibody was not included for ethical reasons.

**Film Autoradiography for Distribution of Radionuclide Activity.** Autoradiography was performed using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). Tissue sections were covered with Saran wrap and overlaid with film alone or with an enhancer screen for exposure durations of between 1 and 14 days. Films were developed in a Kodak RP X-OMAT processor (Eastman Kodak). Film images were digitized using the Nikon Coolsnap (Nikon Electronic Imaging, Melville, NY) and compared to the digitized 35-mm Ektachrome film images of adjacent tissue sections immunostained with mAb MX35. Coregistration of the serial sections permitted visualization of the radiolabeled antibody activity over regions of tumor cells and nontumor tissue.

**Autoradiography Using Storage Phosphor Screens for Distribution of Radionuclide Activity.** For the analysis of storage phosphor screen images, either a model GS-250 (100-μm pixel resolution) or model GS-350 (85-μm pixel resolution) Molecular Imager system (Genetics Systems Division, Bio-Rad Laboratories Inc., Hercules, CA) connected to a Macintosh Quadra 840AV computer (Apple Computers Inc., Cupertino, CA) was used. The storage phosphor screen (type BI) is fabricated from strontium sulfide commingled with elemental cerium and samarium (16). The interaction of ionizing radiation with the storage phosphor screen excites electrons into the conduction band, from which they fall into electron traps. Quantification of the number of filled traps is proportional to the amount of energy deposited in the screen. The detector signal is read out using an externally applied scanning laser (pulsed IR diode at 910 nm), which scans the screen, releasing the electrons from their traps pixel by pixel. The nominal resolution is limited by the focal spot of the laser. The scan itself possesses a greater intrinsic resolution. The process of electron de-excitation results in the emission of fluorescent photons, which are

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collected in a fiber optic pipe and counted using a photomultiplier tube. The resultant signal is processed using an analog-to-digital converter, which provides the final 10–16-megabyte image at 16 bits/pixel. The phosphor image data were analyzed on a Power Macintosh 7100/66 (Apple Computers, Inc.) using Molecular Analyst/Macintosh (version 2.0) data analysis software (Bio-Rad Laboratories, Inc.) and the public domain NIH Image (Version 1.55) program (written by Wayne Rasband, NIH), which is available from the Internet.

Tissue sections were covered with Saran wrap and then clamped against a storage phosphor screen in a light-tight cassette for a preliminary exposure duration of 24 h and a second exposure duration of 4–16 days. The storage phosphor screen was erased after image readout by exposure to IR light for 15 min and erased a second time immediately before exposure to the samples.

**Calibration of Storage Phosphor Screen Using Radionuclide Standards.** A storage phosphor screen image consists of a two-dimensional array of intensities. The conversion of these intensities into specific activity units requires calibration of the storage phosphor screen response. The screen response was analyzed with three sets of radiolabeled standard sources. Strips containing graded standards of 14C (RPA.504 and RPA.511) and 125I (RPA.523) for autoradiographic calibration were purchased (Amersham Laboratories, Buckinghamshire, England). The commercial 125I standards embedded in polymer were quoted at 20-μm tissue equivalent thickness. A set of 131I standards was made by dilution of a stock solution containing a known activity of 131I as follows. Eosin Y (1% alcoholic solution; Polysciences, Inc. Warrington, PA) was added to the radioactive solution, which was then mixed with the OCT compound until a uniform coloration was achieved. The samples were weighed and counted, and the relative specific activities of the two blocks were determined to be 0.536 μCi/g and 4.680 μCi/g, respectively. The two 125I standards were sectioned at 6-μm thickness and dried onto microscope slides in the same way as the tissue sections prior to exposure to the screen for 24 h. The response of the phosphor screen was determined to be 1814.5352, and 1003 cpd per pixel for 131I, 125I, and 14C, respectively, for a source of 1 μCi/g specific activity. The higher sensitivity of the storage phosphor screen means that exposure times are typically between 5 and 10 times shorter than film for the same image quality (17). The 14C standards were placed alongside the iodine standards to evaluate the constancy of the storage phosphor screen over a long period.

The phosphor screen images were analyzed retrospectively for all patients after ascertaining the phosphor screen response, i.e., cpd per pixel per unit specific activity, as a function of exposure duration from standards. 131I, 125I, and 134Cs standards were exposed alongside sections of both normal and tumor tissues for patients 5 and 6. This allowed the unknown activity distribution in the biopsied specimen to be determined by direct scaling from the known activity of the standards and the ratio of the radionuclide response. The validity of our approach to patients 2, 3, and 4, who were studied prior to the simultaneous exposure to standards, was verified by estimating the specific activity for patients 5 and 6 using both methods. The ratios between the values obtained by the new method (involving simultaneous exposure to standards) to the previous method (applying the known response and fade characteristics of the phosphor screen) were 0.86 and 0.95 for cases 5 and 6, respectively.

The storage phosphor screen exhibits a slow signal fade during the signal acquisition time. Thus, for each sample exposure time, it was required to convolute the signal accumulation with the signal fade. The fade characteristics were determined by repeatedly exposing the screen to the standards for the same 1-h duration and varying the interval before reading the screen, from immediate up to 14 days. The correction factor to account for signal fade \( F \) for a specimen exposure of duration \( t_s \) is given by the convolution integral \( \int_0^{t_s} e^{-t_s \lambda_F} \cdot e^{-\lambda_F t} dt \), where \( \lambda_F \) is the rate of signal loss attributed to fade and \( \lambda_p \) is the physical decay constant for the radionuclide. This integral accounts for the variable amount of fade during the continuous phosphor screen exposure; i.e., for an 8-day exposure, the counts on day 1 fade for 7 days, those on day 2 fade for 6 days, and so on. Solving the integral, one obtains the following:

\[
F = e^{-\lambda_F t_s} \left[ 1 - e^{-\lambda_p t_s} \right]/(\lambda_p - \lambda_F)
\]

The rate constant \( \lambda_p \) for phosphor screen fade was found to be 0.0967/day. Inserting the decay constant \( \lambda_p \) for 131I, the above equation becomes

\[
F = e^{-0.0962 t_s} \left[ 1 - e^{-0.0105 t_s} \right]/0.0105,
\]

where \( t_s \) is the time in days the phosphor screen is exposed to an 131I-labeled specimen.

**Estimation of Dosimetry using Storage Phosphor Screens.** The radiation dose is directly proportional to the cumulative specific activity of the radiolabeled antibody in the tumor. Autoradiographic images provide information about the activity in the tissue at only a single time. Because these patients only had minimal residual disease, it was not possible to obtain tumor clearance data from nuclear medicine gamma camera scans. Therefore, the storage phosphor screens were only able to accurately measure the specific activity of the radiolabel in the tumor microdeposits relative to the surrounding normal tissue or nontumor specimens. Parallel studies, performed with the same radiolabeled 131I-labeled mAb MX35 F(ab′)_2 in a human ovarian cancer xenograft model (OVCA/3), showed a biological half-life \( (T_h) \) of 15.5 h for both i.v. and i.p. routes of injection (18). This half-life is reasonable for F(ab′)_2 antibodies directed against secreted antigens, as evidenced by biological half-lives reported in patient trials after i.p. injection with other radiolabeled mAbs, e.g., \( T_h = 21 \) h for 111In-mAb OCT125 (19), and \( T_h = 14 \) h for 186Re-mAb NR-CO-02 (20). The mAb MX35 binds to a cell-surface antigen, and it may be that the short biological half-life observed in animal studies is the result of deiodination. Our clinical data suggest a similar clearance in patients. One patient (case 4) was biopsied after only 15 h, compared to the other five patients. On the basis of the ratio of the %ID/g for case 4 relative to cases 1, 3, and 6, which were biopsied at 4 days, we calculated an approximate \( T_h \) of 18.4 h for the patients studied. The similarity of this value with data reported by other investigators shows internal consistency with our patient data. Unfortunately, true clearance data from microscopic tumor cell foci are not currently possible in situ. Assum-
estimated the doses based on an assumed dependence of these estimates upon the assumed to use unity for the absorbed fraction as 13-particles, it is recommended by the MIRD committee (21). For nonpenetrating radiations, such \( n_1E_1 \) \( \times 10^4 \) deposit containing less than 1 g of tumor cells, the value of the second term consists of the product between the total energy \( n_1E_1 \) \( \times 41. \)

bracket the range of possible doses and to show the strong average from other clinical trials (4-8). These values were to correspond to the murine data (18), and a \( T_9 \) are published by Humm (22) and Goddu et al. 

using the same biological half-life for patients and animals would result in a \( T_e = T_{e1}T_{e2}(T_{n1} + T_{n2}) \) of 14.3 h for \( ^{131}I \) and 15.3 h for \( ^{125}I \), and the physical half-life \( T_9 \) \( 8.04 \) days for \( ^{131}I \) and 60 days for \( ^{125}I \). In this clinical trial, the principal radionuclide delivered was either \( ^{125}I \) (cases 1 and 2) or \( ^{131}I \) (cases 3–6), and the calculations that follow estimated the specific activity in micrometastases for the appropriate radiolabeled mAB MX35 F(ab')2. To estimate radiation dose to micrometastatic foci required a calculation of the cumulative specific activity (area under activity-time curve). Therefore, the activity versus time must be back extrapolated from measurements of the whole biopsy, to a time \( t_e \). Using the assumption of a monoexponential clearance rate \( C_0 = C(t)e^{-\lambda t} \), where \( C_0 \) and \( C(t) \) are the specific activity in \( \mu \)Ci/g at the time of mAB infusion and surgery, respectively, \( t \) is the time interval, and \( \lambda = 0.693/T_e \). In this study, we estimated the doses based on an assumed \( T_{n1} \) of 15.5 h, corresponding to the murine data (18), and a \( T_n \) at 24 h, a simplified average from other clinical trials (4–8). These values were to bracket the range of possible doses and to show the strong dependence of these estimates upon the assumed \( T_{n1} \). The radiation absorbed dose is given by the integral of the specific activity \( C_0f_{e^{-\lambda t}} \) over time multiplied by 2.13 \( \Sigma(nE_1)\phi_e \). The second term consists of the product of the total energy \( \Sigma(nE_1) \) released by radionuclide emission, the fraction of emission energy absorbed within the tumor \( (\phi) \), and 2.13, which is a unit conversion factor. For \( ^{131}I \), the sum of the \( \beta \)-ray energy emitted per decay \( \Sigma(nE_1) = 0.187 \text{ MeV} \) multiplied by 2.13 equals 0.398 g-cGy/\( \mu \)Ci-h. For nonpenetrating radiations, such as \( \beta \)-particles, it is recommended by the MIRD committee (21) to use unity for the absorption fraction \( \phi \). For a micrometastatic deposit containing less than 1 g of tumor cells, the value of \( \phi \) is less than 1. The absorbed fractions for several radionuclides, including \( ^{131}I \), are published by Humm (22) and Goddu et al. (23). For a 100-\( \mu \)m micrometastatic lesion, \( \phi \) is equal to 0.17; i.e., 17% of the energy emitted within the lesion is deposited locally.

RESULTS

From a study evaluating the localization of radiolabeled mAB MX35 F(ab')2 in patients with ovarian carcinoma, specimens were taken during second-look surgery after antibody administration 1–5 days earlier. The activity determined by well scintillation counting of the whole-tissue specimens was compared with storage phosphor screen autoradiography of tissue sections (24). Biopsied specimens from six patients were analyzed (Table 1). All tumors expressed MX35 antigen as determined by immunohistochemical analysis. In total, 19 normal tissue biopsies and 16 biopsies containing tumor cell foci were evaluated in the laboratory.

### Table 2 Summary of %ID/g in tumor, normal tissue specimens, and serum for radiolabeled mAB MX35 F(ab')2 by well scintillation counting of whole biopsies

<table>
<thead>
<tr>
<th>Time of Case surgery</th>
<th>%ID/g for (^{131}I)-labeled mAB (( \times 10^{-3} ))</th>
<th>%ID/g for (^{125}I)-labeled mAB (( \times 10^{-3} ))</th>
</tr>
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<tbody>
<tr>
<td>Tumor</td>
<td>Normal tissue (fat)</td>
<td>Tumor (fat)</td>
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<tr>
<td></td>
<td>(( \times 10^{-3} ))</td>
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<tr>
<td></td>
<td>Serum</td>
<td>Tumor:Serum</td>
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<tr>
<td></td>
<td>(( \times 10^{-3} ))</td>
<td>(^{131}I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(^{131}I)</td>
</tr>
<tr>
<td>1 Day 4</td>
<td>1.35-3.18</td>
<td>0.35-1.26</td>
</tr>
<tr>
<td>2 Day 5</td>
<td>8.71</td>
<td>0.54-1.71</td>
</tr>
<tr>
<td>3 Day 4</td>
<td>0.52-1.33</td>
<td>0.49-0.53</td>
</tr>
<tr>
<td>4 Day 5</td>
<td>22.35</td>
<td>1.27-7.27</td>
</tr>
<tr>
<td>5 Day 6</td>
<td>7.98</td>
<td>0.02-1.17</td>
</tr>
<tr>
<td>6 Day 7</td>
<td>0.83-1.30</td>
<td>0.52-0.70</td>
</tr>
</tbody>
</table>

* Two mg of mAB MX35 F(ab')2 were labeled with \(^{131}I\) and \(^{125}I\) (cases 1–5). In case 6, radiolabeled mAB was mixed with 8 mg of cold mAB MX35 F(ab')2.

* mAB was delivered by i.v. route (cases 1–3 and 6) or by i.p. route (cases 4 and 5) between 15 hours to 5 days prior to surgery.

* The amount of radionuclide activity administered per patient ranged from 2 to 15 mCi for \(^{131}I\) and 2 to 5 mCi for \(^{125}I\).

* Whole blood removed during surgery was centrifuged, serum was aspirated, and 1 ml of serum was weighed and counted in a well scintillation counter.

* Tumor:fat ratios are presented because fat (values in parentheses in columns 4 and 7) was the only tissue consistently available for all six cases examined. The range of normal tissue data could be used to calculate the tumor:average normal tissue ratios.
Fig. 1  A, tumor biopsy from patient 2 with a serous ovarian carcinoma following administration of radiolabeled mAb MX35 F(ab')2 (5 days earlier) by an i.v. route. Indirect immunoperoxidase staining with mAb MX35 of carcinoma at high power (a; bar, 100 μm) and low power (b; bar, 2 mm). Adjacent tumor sections of digitized film image at low power (c) and storage phosphor screen image at low power (d). Note the strong immunoperoxidase staining of tumor cell clusters and colocalization of radiolabeled mAb to the tumor. In contrast, immunoperoxidase staining of the normal peritoneal wall biopsy at low power (e; bar, 2 mm) and uptake of radiolabeled mAb on the corresponding digitized film image (f) and storage phosphor screen image (g) were not detected. B, para-aortic lymph node from patient 5 with a poorly differentiated ovarian carcinoma following administration of radiolabeled mAb MX35 F(ab')2 (5 days earlier) by an i.p. route. Indirect immunoperoxidase staining with mAb MX35 at high power (a; bar, 100 μm) and low power (b; bar, 2 mm). Adjacent tumor sections of digitized film image (saturated) at low power (c) and storage phosphor screen image at low power (d). Note the distribution of immunostained tumor cells between the hematopoietic cells and stromal tissue (b, arrowhead) and localization of radiolabeled mAb to the tumor cell area specifically. C, tumor cell clusters in ovary (a–d) and fallopian tube (e–h)
Epithelium and tumor cell clusters show strong immunoperoxidase staining. However, the radiolabeled mAb localizes to the tumor cell clusters on high-power photographs (a–c) and the phosphor screen image (Fig. 1A, d) shows specific targeting in the areas of radiolabeled mAb accumulation. The immunoperoxidase-stained antigen localization shows a variable intensity within the tumor cell foci, and this may account for an apparent heterogeneity of the autoradiographic images. Sections of the adjacent peritoneal wall biopsy are negative (Fig. 1A, e–g).

Fig. 1B illustrates a para-aortic lymph node specimen (case 5) with a micrometastatic tumor cell cluster from a poorly differentiated ovarian carcinoma detected by immunostaining at high power (Fig. 1B, a) and low power (Fig. 1B, b, arrowhead) surrounded by hematopoietic cells. The film image has saturated at the 7-day exposure (Fig. 1B, c). The phosphor screen image has a greater dynamic range and therefore does not saturate (Fig. 1B, d). The 1-day film and phosphor screen images were less intense and showed radionuclide localization only to the specific clusters of tumor cells as detected by immunostaining in Fig. 1B, b (data not shown).

Fig. 1C illustrates an endometrioid-type carcinoma of the peritoneum (case 6). A micrometastatic tumor cell cluster was found near the surface of the ovary (Fig. 1C, a–d), and multiple clusters of tumor cells were detected adjacent to the fallopian tube epithelium (Fig. 1C, e–h). The small lesion in the ovary (Fig. 1C, b, arrowhead) is targeted specifically by radiolabeled mAb MX35 Fab′(a′), as seen in the autoradiographic film and phosphor screen images (Fig. 1C, c and d). Note that the fallopian tube epithelium expresses MX35 antigen along the apical surface (Fig. 1C, e and f), but the radiolabeled antibody localizes preferentially to the tumor cell clusters (Fig. 1C, g and h).

Determination of Specific Activity of Radiolabeled Antibody within Tissue Sections Using Storage Phosphor Screen Analysis. Line profiles were drawn so as to traverse regions of micrometastatic tumor cell foci, which impregnate the regions of normal stromal tissue. The variation in cpd per pixel was relatively uniform throughout normal tissue and in areas of stromal tissue in biopsies containing tumor but exhibited “peak” values at spatial positions corresponding to tumor cell foci, as confirmed by immunoperoxidase staining. For small tumor cell clusters, the peak values are averaged, and for biopsies with multiple, discrete tumor cell foci, a range of values is quoted corresponding to different regions or different tissue sections. Average pixel values are quoted for normal tissues. Rations of the “peak” response (converted to cpd) overlaying the MX35-positive tumor deposits relative to the normal stromal tissue background can differ significantly, as illustrated in Fig.

Immunohistochemical Delineation of Areas of Tumor Cells in Tissue Sections and Comparison with Film and Storage Phosphor Screen Autoradiography. The autoradiographs from X-ray film and storage phosphor screens provide an image of the distribution of radionuclide devoid of its relation to the histology of the tissue section. Coredistribution of phosphor images and/or digitized film images with the digitized 35-mm Ektachrome film images of tissue sections, stained by immunoperoxidase using mAb MX35 for antigen localization, allowed visual assessment of the efficacy of radiolabeled mAb targeting. Film images of tumor and normal tissue sections were available for six cases. Clusters of tumor cells were detected clearly by film visualization in five of six cases; in the other case, single tumor cells and small clusters of <10 tumor cells were detected weakly by film (case 3). Phosphor screen images were analyzed for tumor and normal tissue sections in five cases; single tumor cells and small tumor cell islets did not image clearly in case 3.

Analysis of adjacent tissue sections confirmed the coincidence of the radiolabeled mAb MX35 Fab′(a′)2 to regions of MX35-positive tumor cells. Fig. 1 illustrates the results from three cases comparing film with storage phosphor screen images. Fig. 1A illustrates a small laparoscopy specimen (case 2) with >80% of the specimen having strong MX35 antigen expression on the serous-type ovarian carcinoma cells at high power (Fig. 1A, a). The unstained regions represent stromal tissue in which tumor cell foci are embedded. Coregistration of the image from a tumor section immunostained for antigen localization (Fig. 1A, b) with both the film autoradiographic image (Fig. 1A, c) and the phosphor screen image (Fig. 1A, d) shows specific targeting in the areas of radiolabeled mAb accumulation. The immunoperoxidase-stained antigen localization shows a variable intensity within the tumor cell foci, and this may account for an apparent heterogeneity of the autoradiographic images. Sections of the adjacent peritoneal wall biopsy are negative (Fig. 1A, e–g).

Fig. 2 Determination of specific activity of radionuclide within tumor specimens from patient 4 with an endometrioid ovarian carcinoma following administration of radiolabeled mAb MX35 F(ab′)2 (1 day earlier) by an i.p. route. Three line profiles were drawn to traverse regions of micrometastatic tumor foci that impregnate the regions of normal stromal tissue (left, a–c). The locations of the profiles are indicated by lines in the tumor biopsy (right, a–c). The ordinate is in units of cpd, which can be converted to pCi/g by dividing by the conversion factor 1841 cpd/pCi/g. Bar, 2 mm.
Table 3  Comparison of µCi/g values and tumor:normal tissue (T:N) ratios from biopsied samples analyzed by well scintillation counts versus storage phosphor screen response

<table>
<thead>
<tr>
<th>Case</th>
<th>Biopsy site*</th>
<th>Time of surgery</th>
<th>Well counts†</th>
<th>Phosphor screen response‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Omentum (T)</td>
<td>Day 4</td>
<td>0.090</td>
<td>3.4:1 NA NA</td>
</tr>
<tr>
<td>2</td>
<td>Diaphragm (T)</td>
<td>Day 5</td>
<td>0.027</td>
<td>13.4:1 0.265 10.2:1</td>
</tr>
<tr>
<td>3</td>
<td>Omentum (T)</td>
<td>Day 4</td>
<td>0.142</td>
<td>2.7:1 0.184 2.0:1</td>
</tr>
<tr>
<td>4</td>
<td>Cul de sac (T)</td>
<td>15 h</td>
<td>2.429</td>
<td>3.1:1 23.116 8.7:1</td>
</tr>
<tr>
<td>5</td>
<td>Lymph node (T)</td>
<td>Day 5</td>
<td>0.790</td>
<td>5.5:1 4.179-6.082 17.8-19.7:1</td>
</tr>
<tr>
<td>6</td>
<td>Peritoneum (T)</td>
<td>Day 4</td>
<td>0.228</td>
<td>1.9:1 1.645-1.849 7.2-8.0:1</td>
</tr>
</tbody>
</table>

* The same biopsied samples were evaluated by well scintillation counts (whole specimen) and storage phosphor screen response (tissue sections). T, biopsy site containing tumor; N, normal tissue biopsy adjacent to tumor.
† Well scintillation counter measurements represent total activity per gram from the entire biopsy specimen.
‡ Storage phosphor screen responses disassociate counts arising from radiolabeled antibody localized in tumor cell foci from stroma. The variation in counts per pixel was relatively uniform throughout stromal tissue. Average pixel values are quoted for the normal tissues. Peak pixel values are given for small tumor cell clusters. For some tumor specimens, a range of values could be quoted corresponding to different regions or different biopsy specimens. NA, not available.

2 from case 4. Each profile in counts per pixel was converted to local specific activity in µCi/g from the phosphor screen response determined by standards. Table 3 compares the specific activity (expressed as µCi/g) for mAb MX35 F(ab’)_2 in the same tumor biopsy samples and adjacent normal tissues, measured by storage phosphor screens (tissue section) and well scintillation counter (whole specimen). Estimates of the tumor-specific activity, expressed as µCi/g from the storage phosphor screens, were compared with measurements of the whole biopsied specimen from a well scintillation counter. The µCi/g values for normal tissue ranged from 0.023 to 0.218 by well counts and from 0.026 to 0.341 by phosphor screen response for samples evaluated 4 or 5 days post-antibody injection. In contrast, µCi/g values for corresponding tumor biopsies ranged from 0.142 to 1.197 by well counts and from 0.184 to 0.6092 by phosphor screen response. Similar µCi/g values for tumor biopsies were estimated by both well counts and phosphor response methods for case 2 with >80% tumor cells in the sample and for case 3 with small clusters and individual tumor cells dispersed throughout the sample. However, µCi/g values were significantly higher by the phosphor screen response method in samples with distinct tumor cell foci (cases 5 and 6), as illustrated in Fig. 1, B and C. The tumor:normal tissue ratios were between 1 and 4 times greater using the storage phosphor screen method than well counter measurements (Table 3), but even larger variations of ratios up to 20:1 were observed between tumor cell foci and hematopoietic cells from within the same tissue section (Fig. 1B). This is a consequence of the ability of the phosphor screen to disassociate counts arising from radiolabeled antibody localized in tumor cell foci from the adjacent stroma and to accurately measure high activity tumor foci within a specimen rather than to use a single average value on the whole specimen from well counter measurements.

Estimation of Dosimetry within Tissue Sections Using Storage Phosphor Screen Analysis. Radiation dose estimates for mAb MX35 F(ab’)_2 in the tumor were made assuming hypothetical T_s of 15.5 h (from animal data) and 24 h, respectively. The estimated dosimetry using a T_s of 15.5 h would result in radiation absorbed doses of 465, 110, 554, 10663, and 1109 cGy/mCi injected for cases 2, 3, 4, 5, and 6, respectively, assuming the usual absorbed fraction of 1 for the β-particle emissions of 131I. However, for isolated microscopic volumes, these doses can be significantly reduced due to the loss of β-particle flux from the tumor cell cluster. Also note that the projected tumor doses are very sensitive to small changes in the assumed biological half-life. For example, when T_s = 24 h, the absorbed doses would be 104, 72, 564, 2375, and 361, respectively, for the same patients. These dose estimates appear excessively high compared to values reported from other radiolabeled mAb clinical trials (25, 26). Although these values could be the consequence of an incorrect assumption for T_s, in estimating the cumulative specific activity, this study differs from others, because we are quantifying the activity per gram in microscopic tumor cell volumes. Furthermore, the dose deposited in small microscopic foci of disease depends on the absorbed fraction of energy emanating from the high-energy β-particles of 131I, which is deposited locally (22). The fraction of 131I β-ray energy absorbed locally within a 100 µm-diameter lesion is 0.17 (23). This would reduce the dose per mCi estimates to the micrometastatic foci to 0.17 times the values presented above. The µCi/g determined by the well counter and storage phosphor screen techniques are summarized in Table 3.

DISCUSSION

In this study, we evaluated the radiolabeled antibody uptake of the murine mAb MX35 F(ab')_2 in biopsied samples from patients with epithelial ovarian cancer by well scintillation counting and by autoradiography using storage phosphor screens. Specific localization of mAb in tumor was demonstrated by coregistration of the immunohistochemical staining in areas of tumor cell clusters with autoradiographic film and
posteriorly on the surface of the peritoneum, where it forms a tumor spread, radiolabeled mAb uptake showed specific localization to tissues for 131I and 125I. There was a general relationship between the radiolabeled mAb uptake in the tumor biopsy and both the level and the intensity of the immunohistochemical expression of the MX35 antigen in the corresponding tumor tissue section. Specific localization of mAb in tumor was demonstrated by tumor:normal tissue (fat) ratios ranging from 0.9:1 to 35.9:1 for 131I and from 0.9:1 to 39.0:1 for 125I. Significantly higher tumor:normal tissue ratios were calculated for the two patients given radiolabeled antibody by the i.p. route (e.g., 17.7:1 and 35.9:1 for 131I). These results can be compared to an earlier clinical trial in which mAb MX35 whole IgG was used (8). In that study, tumor samples obtained at surgery (7–20 days post-mAb infusion) showed a mAb accumulation of between 0.3 and 67.0 × 10−4 %ID/g of tissue, and the tumor:normal tissue (fat) ratios ranged from 2.3:1 to 34.4:1. The tumor:normal tissue ratios were not related significantly to mAb dose, the level of immunohistochemical antigen expression, or the interval between mAb infusion and surgery (8). Also, in contrast to the present study, tumor:serum ratios rarely exceeded 1.0 (8).

The storage phosphor screen enabled radiolabeled antibody to be quantified directly relative to tissue histology and immunohistochemistry. The radionuclide uptake evaluated 4 or 5 days post-antibody injection ranged between 5.2 and 223.5 × 10−4 %ID/g of tumor tissues for 131I and between 2.9 and 210.9 × 10−4 %ID/g for 125I. There was a general relationship between the radiolabeled mAb uptake in the tumor biopsy and both the level and the intensity of the immunohistochemical expression of the MX35 antigen in the corresponding tumor tissue section. Specific localization of mAb in tumor was demonstrated by tumor:normal tissue (fat) ratios ranging from 0.9:1 to 35.9:1 for 131I and from 0.9:1 to 39.0:1 for 125I. Significantly higher tumor:normal tissue ratios were calculated for the two patients given radiolabeled antibody by the i.p. route (e.g., 17.7:1 and 35.9:1 for 131I). These results can be compared to an earlier clinical trial in which mAb MX35 whole IgG was used (8). In that study, tumor samples obtained at surgery (7–20 days post-mAb infusion) showed a mAb accumulation of between 0.3 and 67.0 × 10−4 %ID/g of tissue, and the tumor:normal tissue (fat) ratios ranged from 2.3:1 to 34.4:1. The tumor:normal tissue ratios were not related significantly to mAb dose, the level of immunohistochemical antigen expression, or the interval between mAb infusion and surgery (8). Also, in contrast to the present study, tumor:serum ratios rarely exceeded 1.0 (8).

The storage phosphor screen enabled radiolabeled antibody to be quantified directly relative to tissue histology and immunohistochemistry. The radionuclide uptake evaluated 4 or 5 days post-antibody injection ranged between 0.03 to 0.34 μCi/g for normal tissues, from 0.18 to 0.27 μCi/g in small tumor cell clusters dispersed throughout the tissue (cases 2 and 3), and from 1.65 to 6.08 μCi/g in discrete tumor cell foci by the phosphor screen technique. In contrast, a single average value ranged from 0.02 to 0.22 μCi/g for normal tissues and from 0.09 to 1.20 μCi/g in whole, biopsied specimens containing tumor by well scintillation counts. The tumor:normal tissue ratios were between 1 and 4 times greater using the phosphor screen method than well counter measurements, but even larger variations of ratios were observed between tumor cell foci and stromal cells from within the same tissue section. In this study, the ovarian tumors grew as micrometastatic clusters of cells within loose connective tissue or lymphatic spaces, making them accessible targets for antibody uptake. In an earlier study using radiolabeled-mAb MX35 IgG (8), solid ovarian tumors showed autoradiographic film images with localization confined to the outer rim of viable tumor or within areas of necrotic tumor tissue, indicating a problem with antibody penetration. In sum, these observations suggest that the specific activity may be more accurately measured in specimens with microscopic disease.

Our analysis provided a measure of the specific activity of biopsied specimens at one single time point, namely, at the time the specimen was removed and frozen. Because there was no means to determine the specific activity and microdistribution of the radiolabeled antibody before this time, we performed hypothetical calculations of the radiation dose based on assumed effective half-lives for the 131I-labeled mAb MX35 F(ab')2 of 15.5 h (18) and 24 h. Absorbed dose estimates to microscopic tumor cell clusters must also correct for the fraction of local energy absorption. Such corrections to the absorbed dose are possible with the storage phosphor screen approach because it can account for microscopic variations in local energy deposition, which are averaged by well scintillation counting methods.

To improve the radiation dose estimates presented here, it would be necessary to determine the specific activity of the radiolabeled antibody in the tumor at multiple time points. This information is not available for microscopic disease. Griffith et al. (27) proposed a method to implant thermoluminescent dosimeters, mounted at the tip of a catheter into tissue, to directly measure the radiation dose in situ. However, this method would not be readily applicable to microscopic disease, due to the uncertainty of the location of the tumor cells. A method using PET to quantitatively assess mAb localization in situ has been developed and tested with 124I-labeled mAb 3F8 in a patient with glioma (28). High-resolution PET has been used to localize human ovarian cancer in nude rats using 124I-labeled mAb MX35 (29). The high sensitivity of PET may allow the detection of microscopic disease provided the tumor:background ratio is sufficient to produce an adequate image contrast. However, the resolution of current PET scanners in the abdomen is not better than 4 mm (General Electric Advance PET Scanner, Milwau-kee, WI).

When an antibody can be shown convincingly to localize to micrometastatic tumors, epithelial ovarian cancer affords an ideal opportunity to use the antibody or antibody conjugates for therapy. The specific targeting of mAb MX35 F(ab')2 to micrometastatic disease as shown in this study demonstrates the potential of this radiolabeled antibody conjugate for such a therapeutic trial. The ability to target minimal residual disease embedded in tissue stroma may be a significant rationale for treating patients with refractory or recurrent ovarian cancer with radioimmunotherapy. Small tumors may be more uniformly accessible to mAb and will require lower doses of radiation to eradicate than bulky disease. Ovarian cancer often spreads superficially on the surface of the peritoneum, where it forms small tumor foci within the peritoneal cavity. Extraperitoneal metastases, other than spread to local lymph nodes, are rare. Administration of antibody through an i.p. route, therefore, provides an optimal mode for the treatment of this disease. An additional advantage of i.p. administration is that, although small tumors may be undervascularized, they will still be accessible to radiolabeled antibody by diffusion from the peritoneal fluid.

This study has demonstrated (Figs. 1 and 2) that mAb MX35, in its F(ab')2 form, localizes avidly to the micrometastatic ovarian carcinoma deposits within the peritoneal cavity. Well scintillation counter techniques may underestimate the radiation dose to microscopic tumor cell clusters, because the activity per gram is averaged uniformly over tumor and stromal cells. Storage phosphor screens offer a means of measuring the accumulation of radiolabeled antibody activity within small tumor cell foci more accurately. Preliminary dose estimates...
performed in this study suggest a therapeutic potential for the antibody in patients with minimal residual disease.

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