Measurement of Local $M_1$, 97,000 and 250,000 Protein Antigen Concentration in Sections of Human Melanoma Tumor Using in Vitro Quantitative Autoradiography


ABSTRACT

An assay method that uses $^{125}$I-labeled monoclonal antibody (MoAb) and in vitro quantitative autoradiography was developed to determine the local concentration of tumor-associated antigens in tissue sections. Human melanoma biopsy specimens were evaluated for the expression of the $M_1$, 97,000 and 250,000 protein antigens using MoAb-96.5 and MoAb-9.2.27, respectively. Tissue sections were incubated in solutions of increasing concentration of $^{125}$I-labeled MoAb with or without an excess of unlabeled antibody. Quantitative autoradiography was performed on the sections and compared with $^{125}$I standards to determine tumor-bound radioactivity and calculate bound pmol of MoAb per g of tumor. The total binding, nonsaturable binding, and specific binding of $^{125}$I-labeled MoAb to tumor were then computed. Specific binding of MoAbs to tumor tissue was saturable in all antigen-positive tumors. The maximal concentration of specific binding of antibody to tissue ($B_{max}$) represented the tissue antigen concentration. Estimates of the $K_s$ of antigen/antibody binding were also made. The reliability of the measurements was confirmed by testing sections from mixtures of antigen-positive and antigen-negative cells.

INTRODUCTION

A number of MoAbs directed against tumor-associated antigens have been used for radioimaging and therapy of cancer (1–11). Implicit in this use of monoclonal antibodies is that they will preferentially localize at sites where antigen is expressed. A significant quantity of tumor antigen is therefore necessary for successful targeting with radiolabeled MoAb.

At present, immunocytochemistry is the standard technique to study the expression and distribution of antigens in histological sections of tumors (12, 13). Although this technique allows visualization of antigens and cell components, it gives only a semiquantitative assessment of antigen concentration. Furthermore, these results are dependent on the judgment of the individual reading the histological sections. Because antigen/antibody interactions depend on the concentrations of the two components, it would be useful to know the actual molar concentrations of antigen in tissue. The purpose of the present study was to develop a quantitative method for measuring antigen concentration in histological sections of tumor.

We evaluated a group of malignant melanoma biopsy specimens using antibodies that were specific for p97 and p250 cells. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: MoAb, monoclonal antibody; $B_{max}$, maximal binding; $K_s$, affinity constant; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CS, chicken serum; p97, M, 97,000 protein (p250 defined similarly); PAP, peroxidase-antiperoxidase; ABC, avidin-biotin complex.

Surface antigens. Increasing concentrations of $^{125}$I-labeled monoclonal antibodies were used to saturate tumor antigen sites. The amount of radiolabeled antibody bound to tissue sections was determined with quantitative autoradiography.

MATERIALS AND METHODS

Monoclonal Antibodies: Radiolabeling and Quality Assessment. Three monoclonal antibodies were used for this study. Two of these antibodies, MoAb-96.5 and MoAb-9.2.27 (generously supplied by Hybritech, Inc., San Diego, CA), were specific for human malignant melanoma. The MoAb-96.5 (IgG2a; Hellstrom) reacts with the glycoprotein antigen, p97, whereas antibody 9.2.27 (IgG2a; Morgan and Reisfeld) is specific for the proteoglycan antigen, p250 (14, 15). Both p97 and p250 are cell surface antigens (4, 16). In addition, MoAb-2-135 (Damon Biotech, Needham Heights, MA), also an IgG2a, was used as a negative control antibody in these studies. MoAb-2-135 is specific for a human B-cell lymphoma idiotype and does not react with normal human melanoma or the melanoma cell lines used in this study.

The antibodies were radiiodinated with $^{125}$I (New England Nuclear, Boston, MA) using the chloramine-T method (17). Each mg of protein was reacted with 1 mCi of Na$^{125}$I and 12.5 $\mu$g of chloramine-T (Fisher Scientific, Fairlawn, N.J.). After 2 min, the reaction was stopped by the addition of 43.8 $\mu$g of sodium thiosulfate (Fisher Scientific). The radiolabeled protein was purified from unbound iodide by Sephadex G-25 (Pharmacia, Inc., Piscataway, NJ) chromatography. In all instances, greater than 95% of the radioactivity in the purified product was precipitable with 10% trichloroacetic acid (Fisher Scientific). The specific activity ranged from 0.32 to 0.69 mCi/mg. For the $^{125}$I-labeled 9.2.27 preparations, from 0.57 to 0.86 mCi/mg. For the $^{125}$I-labeled 2-135 preparations, 0.32 to 0.69 mCi/mg.

Immunoreactivity of the radiolabeled monoclonal antibodies was determined with a cell binding assay in which 5 $\mu$g of labeled antibody were reacted with antigen-positive (p97 and p250) human melanoma cells (FEMX II; Frederick Cancer Research Center, Frederick, MD) in concentrations ranging from 0.25 to 8 million cells in 0.2 ml. Nonspecific binding was assessed by adding 25 $\mu$g of unlabeled antibody. Immunoreactivity equaled the maximal cell-bound counts (corrected for nonspecific binding) expressed as a percentage of the total counts added (18). The $^{125}$I-labeled MoAb 96.5 preparation immunoreactivity ranged from 65% to 74%. For the $^{125}$I-labeled 9.2.27 preparations, the specific activity ranged from 0.68 to 0.89 mCi/mg, and for the $^{125}$I-labeled 2-135 preparations, from 0.32 to 0.69 mCi/mg.

From these tumors, consecutive S-100 frozen sections were cut in a cryostat (Cryotome; EM Corporation, Cambridge, MA). Immunocytochemistry was performed on the sections using antibodies that were specific for p97 and p250 cell surface antigens. Increasing concentrations of $^{125}$I-labeled monoclonal antibodies were used to saturate tumor antigen sites. The amount of radiolabeled antibody bound to tissue sections was determined with quantitative autoradiography.

Tissue Preparation. Frozen biopsy specimens from patients with malignant melanoma were provided to the NIH by referring physicians. From these tumors, consecutive 8-μm frozen sections were cut in a cryomicrotome corresponding to the largest cross-sectional area of the tumor. The sections were then fixed in a solution of 0.25% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in PBS (pH 7.4) for 20 min and then washed in PBS for another 20 min. For histological compar-

5 Information on the binding of MoAb 2-135 to normal tissues was provided by Damon Biotech, Needham Heights, MA, and the Frederick Cancer Research Center, Frederick, MD.

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Saturating Study. The tissue sections used for the saturation study were organized into three groups: (a) those used to study the total binding of specific antibody; (b) those used to study nonsaturable binding of specific antibody; and (c) those used for negative control studies. Consecutive sections of tissue cut from the frozen block were assigned so that the first section was placed in the first group, the second section was placed in the second group, and the third section was placed in the third group. This sequence was repeated several times.

Group 1 (total binding of specific antibody) sections were incubated (30 min, 22°C) in a solution containing 2% BSA and 10% PBS (BSA/CS in PBS) to reduce nonspecific binding of radiolabeled antibody. Individual sections were then incubated (60 min, 22°C) in individual solutions of 125I-labeled specific antibody. The concentration of 125I-labeled antibody in these solutions ranged from 1.4 to 126 nanomolar/liter.

Group 2 (nonsaturable binding of specific antibody) sections were preincubated in a humidity chamber (30 min, 22°C) with 75 μl of a 1-mg/ml solution of unlabeled specific antibody in PBS. These slides were then further incubated (5 min, 22°C) in a PBS wash to remove excess unlabeled antibody. Following an incubation (30 min, 22°C) with BSA/CS in PBS, individual sections were incubated in individual solutions where concentrations of 125I-labeled antibody ranged from 1.4 to 126 nanomolar/liter. In addition, the solutions contained 0.6 μmol/liter of unlabeled specific antibody.

Group 3 (total binding of the negative control antibody) sections were initially incubated (30 min, 22°C) in BSA/CS in PBS solution. Individual sections were then incubated in individual solutions of 125I-labeled antibody 2-135. The concentration of these solutions again ranged from 1.4 to 126 nanomolar/liter. After this series of incubations, slides from all three groups were washed in PBS (30 min, 22°C) and dehydrated in successive baths of 70% and 100% ethanol.

Quantitative Autoradiography. The general features of quantitative autoradiography and the preparation of autoradiographic standards have previously been reported (19–21). Human serum albumin (The American Red Cross, New York, NY) was labeled with 125I using the chloramine-T method described above. Serial dilutions were prepared ranging from 0.1 to 50 μCi/ml. Two hundred mg of gelatin (Sigma Chemical Co., St. Louis, MO) were added to 0.8 ml of each dilution in polypropylene tubes which were then capped and heated to 50°C for 5 min until the gelatin melted. Aliquots of the gelatin suspensions were then weighed and counted in a gamma counter (United Technologies-Packard, Downers Grove, IL) so that the μCi per g of suspension could be determined. The remainder of each suspension was frozen (forming gelatin sticks) in precooled liquid dichlorodifluoromethane (VWR Scientific Co., St. Louis, MO) and stored in -40°C. Eight-μm sections were cut from each frozen gelatin stick and mounted on glass coverslips. These were then used as the autoradiographic standards.

The three sets of tissue sections, along with the 125I standards, were placed in a film cassette with Kodak SB5 X-ray film. After 2 days of exposure, the film was photographically processed. The X-ray film autoradiographic images were then digitized using a scanning microdensitometer (P-1000 HS; Optronics International, Inc., Chelmsford, MA) and PDP 11/60 computer (Digital Equipment Corp., Maynard, MA).

Absorbance readings within 50- x 50-μm areas were used to reconstruct images in a 512- x 512-pixel matrix. The absorbance measurements from images of the 125I standards were plotted against the respective μCi/g in each standard. A polynomial fitting of these data provided a standard curve (21). The reconstructed (digitized) images (as well as the autoradiographic images on film) were similar to the macroscopic architecture of the tissue sections. By comparing the autoradiographic images to the images of adjacent sections stained with hematoxylin and eosin, tumor areas were defined and regions of interest were drawn over these areas. The mean absorbance values of the selected regions were obtained, and the μCi/g of tumor were determined from the standard curve. Using the specific activity values for each 125I-labeled antibody preparation, the μCi/g values were converted to pmol of immunoglobulin bound per g of tissue.

Analysis of Immunoglobulin Binding Data. The results of the saturation study were analyzed using MLAB, a mathematical modeling program (22). The data from the tissue sections in the nonsaturable binding group (Group 2) were fitted using the function

\[NSB = aL\]

where \(NSB\) is the binding of the 125I-labeled specific MoAb to tissue (expressed as pmol/g) in the presence of an excess of unlabeled antibody, and \(L\) is the concentration of 125I-labeled MoAb in the incubation medium (expressed as nmol/liter). The parameter \(a\) is the slope of the nonsaturable binding curve and was determined by simple linear regression analysis. Using nonlinear least-squares analysis, the data from the total binding (Group 1) tissue sections, incubated with the specific antibody, were fitted with the function

\[TB = (B_{max} \times L)/(K_d + L) + aL\]

where \(TB\) is the total binding of 125I-labeled MoAb to tissue (expressed as pmol/g), \(B_{max}\) is the maximal amount of 125I-labeled MoAb that can specifically bind to tissue (pmol/g), \(L\) is the concentration of 125I-labeled MoAb in the incubation medium (nmol/liter), \(K_d\) is the dissociation constant of the antigen/antibody reaction, and \(a\) is the slope of the nonsaturable binding curve.

The parameters \(B_{max}\) and \(K_d\) were estimated by the computer program which iteratively adjusted their values to obtain the best fit of the data (minimize the sum of square residuals). In addition, the program provided measures of the uncertainty in these parameter estimates (standard error, dependency value). Since total binding \((TB)\) equals specific binding plus nonsaturable binding, the program computed the specific binding and also plotted the curve for this function. The constant, \(K_d\), was calculated as \(K_d = 1/K_a\). Binding data from Group 3 sections incubated with negative control antibody were fitted with a linear function.

Cell Standards. Antigen-positive and antigen-negative cells were used for cell standards. The human melanoma cell line 2669 Cl-13, which expresses the p250 antigen, was provided by Oncogen, Inc. Two murine melanoma cell lines M2-K1735 and M2-SVp97.B/F1 were also obtained from Oncogen, Inc. (Dr. J. Brown). Cell line M2-SVp97.B/F1 was transfected with the p97 expressing plasmid and contains this antigen on its cell surface. M2-K1735 expresses neither the p97 nor the p250 antigen and does not significantly react with monoclonal antibodies 96.5, 9.2.27, or 2-135.

Mixtures of antigen-positive (M2-SVp97.B/F1 or 2669 Cl-13) and antigen-negative (M2-K1735) cells were prepared in 20% gelatin and then frozen in precooled liquid dichlorodifluoromethane to form cell sticks. The total cell number per g was held constant with the percentage of antigen-positive cells varying from 0% to 100%. Eight-μm frozen sections were then cut from each cell stick and processed in the same manner as the tissue sections. In order to assess the variability of the assay procedure, duplicate sections of the cell standards were mounted on the same slide and processed with the saturation study technique.

Immunoperoxidase Staining. Sections consecutive to those used for autoradiography were processed for immunoperoxidase staining. The PAP method was used to identify p97 antigen, and the ABC method was used for the studies of p250 antigen (12, 13). The intensity of the chromogen reaction was graded 0 to 4 plus for each slide (16, 23).

Statistics. In the cell standard experiments, the antigen-positive cell concentration and the \(B_{max}\) values were compared using Pearson’s coefficient of correlation. The results from immunoperoxidase staining and in vitro autoradiography were compared using nonparametric correlation (Spearman’s rank correlation) (24).

RESULTS

Saturating Study. Table 1 shows the results of the saturation study for 8 human melanoma tumors. Representative binding curves obtained from the assays are shown in Fig. 1. Both total and nonsaturable binding of 125I-labeled MoAb, in this case 9.2.27, increased with the increasing concentration of labeled...
Table 1 Maximal binding ($B_{\text{max}}$) and affinity constants of monoclonal antibody (96.5 and 9.2.27) binding to sections of human melanoma tumors

<table>
<thead>
<tr>
<th>Case</th>
<th>96.5 (pmol/g)</th>
<th>$K_a$ (L/M)</th>
<th>9.2.27 (pmol/g)</th>
<th>$K_a$ (L/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Undet.</td>
<td>59.4</td>
<td>5.35 x 10^9</td>
<td>Undet.</td>
</tr>
<tr>
<td>2</td>
<td>12.2</td>
<td>5.12 x 10^4</td>
<td>117.6</td>
<td>2.62 x 10^9</td>
</tr>
<tr>
<td>3</td>
<td>89.5</td>
<td>1.74 x 10^4</td>
<td>12.9</td>
<td>7.48 x 10^4</td>
</tr>
<tr>
<td>4</td>
<td>59.0</td>
<td>0.93 x 10^4</td>
<td>293.3</td>
<td>2.21 x 10^9</td>
</tr>
<tr>
<td>5</td>
<td>19.8</td>
<td>4.00 x 10^4</td>
<td>74.2</td>
<td>6.70 x 10^4</td>
</tr>
<tr>
<td>6</td>
<td>117.8</td>
<td>4.53 x 10^4</td>
<td>7.3</td>
<td>2.10 x 10^6</td>
</tr>
<tr>
<td>7</td>
<td>Undet.</td>
<td>28.8</td>
<td>7.13 x 10^4</td>
<td>Undet.</td>
</tr>
<tr>
<td>8</td>
<td>Undet.</td>
<td>4.4</td>
<td>0.25 x 10^9</td>
<td>Undet.</td>
</tr>
</tbody>
</table>

*a L/M, liters/mole.
*b Undet., undetectable; Indeterm., indeterminate.

Fig. 1. Representative binding curves obtained from the assay performed on an antigen-positive tumor. $^{125}$I-9.2.27 concentration (nmol/liter) on the x axis; binding of $^{125}$I-9.2.27 to tumor (bound pmol/g) on the y axis. Total binding of $^{125}$I-9.2.27 (•), nonsaturable binding of $^{125}$I-9.2.27 (A), specific binding of $^{125}$I-labeled antibody 9.2.27 (O). Nonsaturable binding was determined using excess unlabeled antibody. The binding isotherm was obtained by subtracting the nonsaturable binding values (0.05% to 0.30%) (Table 2).

The $B_{\text{max}}$ values could be determined for all tumors except in three instances (Table 1, Cases 1, 7, and 8), where the total binding and nonsaturable binding curves were nearly identical and both sets of data were best fit by linear functions. In these instances specific binding could not be demonstrated. The binding curves from one of these tumors are shown in Fig. 2. It was concluded that these three tumors expressed a very low or negligible amount of p97 antigen.

The range of the $K_a$ values (Table 1) was approximately 5-fold. For Specimens 1 and 8, the $K_a$ values for the binding of antibody 9.2.27 could not be accurately estimated. In the first case, the uncertainty was caused by a lack of data points in the region of the $K_a$. With Specimen 8, the uncertainty was caused by the low concentration of p250 antigen and the subsequent high correlation of $B_{\text{max}}$ and $K_a$.

The binding of $^{125}$I-labeled 2-135, the negative control antibody, to tissue sections was similar to binding of specific antibody to antigen-negative tumors. It was also similar to results with antigen-positive tumors that were incubated with $^{125}$I-specific antibody and an excess of unlabeled specific antibody.

 Digitized autoradiographic images from tissue sections incubated with $^{125}$I-labeled antibody 96.5 are shown in Fig. 3. The same preparation and the same amount of $^{125}$I-labeled antibody were used for both sections, but one (Fig. 3A) was incubated with labeled antibody alone, whereas the second (Fig. 3B) was incubated with both labeled and excess unlabeled antibody. In general and as evident in Fig. 3, images from the total binding studies (Fig. 3A) showed a heterogeneous pattern of activity, probably reflecting nonuniform antigen expression in tumors. In contrast, images from the nonsaturable binding studies (Fig. 3B) showed a very uniform distribution of activity.

Cell Standards. In order to confirm that the $B_{\text{max}}$ values were linearly related to antigen concentration, mixtures of antigen-positive and antigen-negative cells in gelatin were prepared as cell standards and studied using the saturation technique. Fig. 4 shows the binding of $^{125}$I-labeled MoAb 96.5 to sections of antigen-negative cells (M2-K1735). No significant difference was observed between total and nonsaturable binding. Similar results were obtained using $^{125}$I-labeled 9.2.27. Sections of cell standards with the percentage of antigen-positive cells (M2-SVp97.B/F1 cells for p97 antigen, 2669 Cl-13 cells for p250 antigen) ranging from 0% to 100% were tested with $^{125}$I-labeled 96.5 and 9.2.27 MoAbs. There was a strong positive correlation between the $B_{\text{max}}$ values and the concentration of antigen-positive cells ($r = 0.98, P < 0.01$ for $^{125}$I-labeled MoAb 96.5.

antibody in the incubation medium. Nonsaturable binding was linearly related to the concentration of antibody and had a mean value of less than 2% of the total amount of $^{125}$I-labeled antibody present. Increasing unlabeled antibody in the individual solutions by a factor of 10 caused only a minimal reduction of the nonsaturable binding values (0.05% to 0.30%) (Table 2). Therefore, the lower concentration (0.6 L/M) was used for determining nonsaturable binding in these studies.

Saturable binding of tissue sections of human melanoma tumors was observed for both of the antimelanoma antibodies, 96.5 and 9.2.27. Because the saturation of $B_{\text{max}}$ value expressed as pmol/g was obtained with a large excess of labeled antibody (enough to yield one antibody molecule per antigenic site), the $B_{\text{max}}$ value also represented the tissue concentration of antigen.
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Fig. 3. A, digitized autoradiogram of a section of human melanoma nodule incubated with 126 nmol/liter of 125I-96.5. The values of bound radioactivity are shown in the color scale on the right. Note the nonuniform distribution pattern of bound antibody. B, digitized autoradiogram of an adjacent section of the same tumor of A. This section was preincubated with 1 mg/ml of unlabeled 96.5 and then incubated with 126 nmol/liter of 96.5 plus an excess (0.6 μM) of cold antibody. Note the uniform distribution pattern of the bound antibody.

Fig. 4. Binding curves obtained from the assay performed on p97-negative cells embedded in gelatin and frozen. 125I-96.5 concentration (nmol/liter) on the x axis; binding of 125I-96.5 to the cells (bound pmol/g) on the y axis. Total binding of 125I-96.5 ( ), nonsaturable binding of 125I-96.5 (A). These binding data were comparable to those shown in Fig. 2 obtained from an antigen-negative tumor.

Fig. 5. Bmax versus the concentration of antigen-positive cells. M, 97,000 protein-positive cells M2 SVp97.B/F1 ( ), M, 250,000 antigen-positive cells 2669 Cl-13 ( ). A strong positive correlation was found between Bmax and cell concentrations (r = 0.98, P < 0.01 for 96.5; r = 0.99, P < 0.01 for 9.2.27).

DISCUSSION

Previous studies have suggested that an important factor in using monoclonal antibodies for radioimmunodetection and cancer therapy is the presence or expression of target antigen at tumor sites (2). In general, tumors without antigen are not specifically targeted. For tumors with antigen, the localization of antibody is directly related to the antigen concentration (2, 25). Furthermore, tumors that have heterogeneous antigen expression will likely receive a patchy distribution of antibody that may reduce the effectiveness of therapy (26, 27). Additional factors that affect binding of antibody to specific tumor antigen are the antigen/antibody affinity (or dissociation) constant and binding to cell line M2 SVp97.B/F1, and r = 0.99, P < 0.01 for binding of 125I-labeled MoAb 9.2.27 to cell line 2669 Cl-13) (Fig. 5).

To further define whether the saturation study and in vitro autoradiography could be reliable in measuring local antigen concentration in tumors, within and between assay variability of the Bmax values was assessed using the cell standards. The coefficient of variation of the Bmax value was 7.2% for duplicate sections on the same slide and 13% for sections on different slides that were simultaneously processed. When slides were processed on different days, the coefficient of variation was 20%.

Tumor Antigen Quantitation: Comparison of in Vitro Autoradiography and Immunoperoxidase Staining. Eight human melanoma tumors were analyzed for the p97 and p250 antigens. Immunoperoxidase staining of the tumors showed a broad range of reactivity with MoAb 96.5 and MoAb 9.2.27. Similarly, the Bmax values obtained by in vitro autoradiography ranged from concentrations too small to be detectable to 293 pmol/g (Table 1). The in vitro autoradiographic measurements of Bmax correlated well with the 0 to 4 plus grading of immunoperoxidase staining. For the p97 antigen, measured with MoAb 96.5, the correlation of the two measurements was r = 0.97, P < 0.05, n = 6. In two tumors the PAP-stained sections could not be evaluated. These tumors were then evaluated using the ABC method and were both negative. Each had undetectable levels of antigen by in vitro quantitative autoradiography. Fig. 6 shows the relationship of the results of immunoperoxidase staining and in vitro quantitative autoradiography for all eight tumors. For the p250 antigen, measured with MoAb 9.2.27, the correlation of the results from the two methods was r = 0.96, P < 0.01 (Fig. 7).
the relationship of the antigen concentration to this constant.

The well-known binding equation, here altered slightly

\[ \frac{B}{F} = \frac{[Ag_g]}{K_a} \]

indicates that a substantial fraction of the antibody will bind to tumor only when the antigen concentration equals or exceeds the equilibrium dissociation constant.\(^6\)

Because of the importance of tumor antigen concentration in effective radioligand imaging and radioimmunotherapy and to enhance our understanding of the factors that affect tumor targeting, we sought a technique that could be applied to tissue sections and that would measure tumor antigen levels in terms of molar concentration. In vitro quantitative autoradiography has been extensively used to measure the binding parameters of hormones (29-32) and neurotransmitters (33-36) to receptor sites in various tissues. Furthermore, internally radiolabeled MoAb has been used to identify the location and quantity of Substance P in nervous tissue (37). To the best of our knowledge, ours is the first study in which quantitative autoradiography has been applied to measure tumor-associated antigens in histological sections. The technique allowed us to quantitate both specific and nonspecific (nonsaturable) binding of monoclonal antibodies to tumor tissue. Furthermore, the saturation analysis provided an estimate of the affinity constant for antigen/antibody binding. Digitized autoradiographic images clearly showed the macroscopic architecture of tumors and

\(^6\) The binding equation as first expressed by Berson and Yalow (28) is: \[ \frac{B}{F} = \frac{K_d[Ag_g] - K_d[B]}{K} \] where \(B\) and \(F\) are the bound and free fractions of radiolabeled antibody, \(K_a\) is the equilibrium association constant, and \([Ag_g]\) is the total antigen concentration. Substituting \(K = 1/K_a\) yields \[ \frac{B}{F} = \frac{[Ag_g]}{K_a - [B]/K_a} \]. When \([B]\) \(\ll K_a\), \(B/F = \frac{[Ag_g]}{K_a}\).

the variation of antigen concentration. Although not done in this study, these digital images could be used to quantify the variations in local antigen expression.

In those tumors that were considered to have measurable quantities of antigen, the specific binding of the monoclonal antibodies was a saturable function. The \(B_{max}\) values, obtained with an excess of labeled antibody, thus represented the tissue antigen concentrations. Using this method to measure antigens that contain multiple copies of an epitope would, of course, yield the molar concentration of that epitope. For tissues with levels of antigen too small to be detected, attempts to fit the total binding data with the total binding equation, described earlier, gave statistically invalid results, and the data best fit a linear function. In these instances, the total binding curve and the nonsaturable binding curve, both linear functions, were nearly identical.

The validity of the in vitro autoradiography technique was confirmed using specific and nonspecific (negative control) antibodies, testing antigen-positive and antigen-negative cells, and by showing that maximal binding to cell standards was linearly related to the concentration of antigen-positive cells. The binding data on antigen-negative cells were comparable to the results obtained with antigen-negative tumors. A further confirmation of the validity of the technique was the strong correlation of \(B_{max}\) values and the results of immunoperoxidase staining.

Several methods have been used to quantify immunocytochemical staining of tissue sections (12). One approach is to grade the degree of staining (e.g., 0 to 4+) (16, 23). These readings, which are visual interpretations, require both judgment and experience of the observer. The discrete steps used in
the grading may also yield data that are skewed to lower or higher values. Although techniques used within an individual laboratory (i.e., primary antibody excess, use of a single chromogenic-producing complex, etc.) usually allow relative comparisons of different antigen/antibody systems, the accuracy of these comparisons is not assured. Densitometry evaluation of tissue staining may provide better comparison, particularly within an individual section, but the quantity of antibody binding to the tissue cannot be determined without a system of standards. The in vitro quantitative autoradiography method used in this study yields numerical data which are not dependent on observer judgment and whose values are a continuous function (as opposed to a step function). Furthermore, the autoradiographic standards allow determination of the amount of antibody bound to the tissue. The results for different tissues, using the same or different antibodies, or from different antigen/antibody systems can thus be directly compared.

Because the macrorautograph used in the new technique has approximately a 100-μm² resolution, conventional immunocytochemistry that can be used to evaluate individual cells will not be replaced by this method (20). However, the ability to measure specific antigen concentration in molar units (e.g., pmol/g or mol/mass of tissue) locally within different tumor regions has specific advantages with respect to mathematical modeling and quantitative analysis of antibody targeting. In addition, the comparatively small variability (<20%) associated with in vitro autoradiography is encouraging. This suggests that the results for different tissues and tumors can be confidently compared.

Another important difference between standard immunocytochemistry and the in vitro autoradiography method relates to the definition of specific binding. Although negative controls are generally used for standard immunocytochemistry, "specific binding" actually represents the "total binding" of antibody to tissue (12). With in vitro autoradiography, nonsaturable binding or nonspecific binding can be directly measured and used to determine specific binding. An interesting observation from our initial work, using the new technique, was that some MoAb preparations with low immunoreactive fractions had high nonsaturable binding values. It has been reported that both of the antibodies used in this study yields numerical data which are not dependent on observer judgment and whose values are a continuous function (as opposed to a step function). Furthermore, the autoradiographic standards allow determination of the amount of antibody bound to the tissue. The results for different tissues, using the same or different antibodies, or from different antigen/antibody systems can thus be directly compared.

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