Quantitation of Human Tumor-reactive Monoclonal Antibody 16.88 in the Circulation and Localization of 16.88 in Colorectal Metastatic Tumor Tissue Using Murine Antiidiotypic Antibodies


ABSTRACT

Detection of administered human monoclonal antibodies in the tissues and circulation of patients requires special reagents to overcome interference by normal endogenous immunoglobulin. A practical approach is the development of antiidiotypic antibodies to the human monoclonal antibody and their application in immunoassays specific for the human monoclonal antibody. Accordingly, antiidiotypic antibodies were made to the monoclonal antibody 16.88, a human IgM class anti-colon carcinoma antibody being developed for applications in antibody-targeted immunotherapy of cancer. Three stable clones were obtained that produced antiidiotypic antibodies reactive with 16.88 but nonreactive with human polyclonal IgM or 16.52, a patient-matched IgM monoclonal antibody with different specificity than 16.88. One antiidiotypic antibody, MID 65, was used in a capture format radioimmunoassay to detect 16.88 in the sera of patients who had received 108-ng doses of unlabeled 16.88 coadministered with trace doses of 125I-16.88. Using this assay it was demonstrated that unlabeled 16.88 antibody and 125I-labeled 16.88 antibody did not differ significantly in blood retention for up to 24 h after administration, the period during which the immunoreactivity of the administered antibody remained over 90%. Indirect microautoradiography using exogenously applied 125I-MID 65 to localize 16.88 in frozen metastatic tumor tissue from patients given 16.88 8 days prior to surgery demonstrated the accumulation of 16.88 in areas of apparently healthy tumor cells. Much less 16.88 was detected in stroma or areas of tumor cell necrosis. The accumulation of antibody in nonnecrotic tumor sites encourages the further development of 16.88 for radioimmunotherapy of colon cancer and provides support for further development of human anticytokeratin monoclonal antibodies for cancer therapy.

INTRODUCTION

In the last decade the development of MCA technology for the in vivo diagnosis and therapy of malignant disease has continued to show progress. Murine MCA to tumor-associated antigens have been applied to cancer detection (1–4) and therapy (5–7) with some success. However, a limitation in the development of this technology is that most patients treated with murine antibodies react to the foreign proteins and develop HAMA responses (8, 9). HAMA reduces the effectiveness of the antitumor therapy, alters the pharmacokinetics of the antibody, and may result in deposition of antibody-cytotoxic agent conjugates in reticuloendothelial tissues leading to possible damage (9). In tumor detection, HAMA results in an erosion of image quality, reduces the efficacy of repeat imaging procedures, and prohibits subsequent immunotherapy. Patients may exhibit allergic reactions to the foreign protein, although severe allergic reactions have been rarely seen.

To circumvent these problems, human MCA to tumor antigens have been developed (10). The human MCA 16.88 is an IgM class antibody that recognizes cytoplasmic determinants of colon carcinoma cells. MCA 16.88 was generated from the peripheral blood lymphocytes of a colorectal cancer patient immunized with an autologous tumor cell-Bacillus Calmette-Guérin vaccine (10, 12). Pomato et al. (11) reported that the antigen recognized by 16.88 (CTAA 16.88) represents altered forms of cytokeratins 8, 18, and 19. The antigen is quantitatively expressed to a greater degree in colon tumors and their metastases than in normal tissues. Preclinical studies with radiolabeled 16.88 demonstrated localization in human colorectal cancer xenografts in nude mice (13, 14). Clinical imaging studies in colorectal cancer patients with 131I-16.88 detected tumor sites in 75% of patients studied and indicated an average retention of antibody in the tumor of 17 days with some lesions detectable as long as 23 days postadministration. MCA 16.88 is retained in patients’ tumor long after normal tissue levels have become undetectable (15).

Pharmacokinetic measurements using 131I-labeled 16.88 must be validated to ensure that the distribution of 131I accurately reflects the distribution of the total antibody injected. This is done by comparing levels of 131I-labeled and unlabeled antibody in the circulation of treated patients. Furthermore, evaluation of 16.88 for use in RIT includes investigation of sites of localization within tumor tissue to determine the proximity of tumor-bound 16.88 to areas of healthy proliferating cells. However, detection of human MCA in human tissues and sera requires the development of novel methods to discriminate between the administered human MCA and endogenous human immunoglobulins. In this paper we describe the development of murine Ab2 that specifically react with 16.88 and the application of Ab2 in the detection of administered 16.88 in patients’ sera and metastatic tumor tissues.

MATERIALS AND METHODS

Production of Ab2 to 16.88. A modification of the immunization protocol of Rosemiller and Kelley (16) also described by Matthew and Sandrock (17) was used. Six- to 8-week-old female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were immunized i.v. and i.p. with 25 μg of pooled polyclonal human IgM (ICN Biomedicals, Inc., Lisle, IL) in PBS followed by three injections of cyclophosphamide (Sigma Chemical Co., St. Louis, MO) in PBS (200 mg/kg) i.p. on days 3, 5, and 7. One week later the mice were bled and their sera were tested for suppression of the anti-human polyclonal IgM response by indirect ELISA against human polyclonal IgM. The suppressed mice were then immunized s.c. with 50 μg of 16.88 in complete Freund’s adjuvant and boosted with 50 μg of 16.88 s.c. in incomplete Freund’s adjuvant 2 weeks later. After 3 weeks, mice were bled and their sera were again...
tested by indirect ELISA against 16.88 to confirm the development of a titer to 16.88. Three days prior to fusion, all mice were boosterd i.p. with 50 μg of 16.88 diluted in PBS. A control group was not preimmunized with IgM but did receive cyclophosphamide, followed by immunization with 16.88. Splenocytes were fused with the NS-1 murine myeloma cell line at a ratio of 3:1, and hybridomas were selected in asarseine/hypoxanthine medium according to the method of Fong et al. (18).

A competitive assay was used to determine whether hybridoma supernatants contained Ab2 by demonstrating inhibition of the binding of horseradish peroxidase-labeled 16.88 to CTAA 16.88-coated wells of a microtiter plate. Supernatants containing Ab2 were then tested for direct binding to polyclonal IgM as well as a patient-matched IgM MCA of different specificity than 16.88 (16.52). Three selected clones (MID 65, MID 95, and MID 268) were subcloned twice by limiting dilution at 0.3 cell/well and then carried as an ascitic tumor in BALB/c x DBA/2 F1 (hereafter called CD2F1; Simonsen Labs.) mice. CD2F1 mice produce a greater amount of ascites and higher concentration of antibody compared to BALB/c mice. Additionally, these mice are more hearty and docile than BALB/c mice. MCA were purified from ascitic fluid using protein A-Sepharose (Bio-Rad, Richmond, CA). Purified MCA (greater than 98% purity by SDS-PAGE analysis) were isoytoped by radial immunodiffusion (The Binding Site, San Diego, CA).

Conjugation of MCA. MCA 16.88 and purified Ab2 were radiolabeled with either 125I or 131I(Dupont NEN, Boston, MA) using the Iodo-Gen method of Fraker and Speck. Iodo-Gen was purchased from Pierce (Rockford, IL). Radiochemical purity exceeded 98% and specificity ranged from 7 to 13 μCi/μg.

MCA 16.88 and purified Ab2 were labeled with N-hydroxysuccinimide biotin (Calbiochem, La Jolla, CA) according to the method of Goding (20) at a molar ratio of 1:50 (IgM:biotin) or 1:75 (IgG:biotin).

Ab2 Competitive ELISA. Flat-bottomed Immulon 2 microtiter plates with 96 wells (Dynatech, Alexandria, VA) were coated with 16.88 (5 μg/ml) overnight at 4°C, blocked (1 h, RT) with Dulbecco’s phosphate-buffered saline containing 5% dry milk (w/v), and washed four times with 1% glycerol/0.05% Tween 20 in deionized water. Either 16.88 standards (0.15 to 10 μg/ml diluted in 10% normal rabbit serum-PBS, v/v) or 10% patient serum in PBS was added to the wells followed by 50 nM of the same or different Ab2 labeled with biotin (5 μg/ml). After incubation for 1 h at RT the wells were washed and ABC conjugated to the biotinylated Ab2 was added (30 min, RT). Plates were washed and color development was achieved with 100 mM 3,3′,5,5′-tetramethylbenzidine (Sigma) in phosphate-buffered saline containing 5% dry milk (w/v), and washed four times with 1% glycerol/0.05% Tween 20 in deionized water. Ab2 at dilutions follow by 50 nM of the same or different Ab2 labeled with biotin (5 μg/ml) was added (30 min, RT) to the assay wells. Plates were washed and color development was achieved with 100 mM 3,3′,5,5′-tetramethylbenzidine (Sigma) in 0.1 M sodium acetate buffer, pH 5.5, containing 0.003% H2O2. The reaction was terminated with 4 N H2SO4. Absorbance was measured at 450 nm. Nonspecific binding was determined using 1% BSA in PBS. An Ab2 produced to an unrelated human IgM MCA, 28A32, (10), was added (30 min, RT). Plates were washed and color development was achieved with 100 mM 3,3′,5,5′-tetramethylbenzidine (Sigma) in 0.1 M sodium acetate buffer, pH 5.5, containing 0.003% H2O2. The reaction was terminated with 4 N H2SO4. Absorbance was measured at 450 nm. Nonspecific binding was determined using 1% BSA in PBS. An Ab2 produced to an unrelated human IgM MCA, 28A32, (10), was used as a specificity control in these studies.

Immunoreactivity Assay for 16.88. 125I-16.88 in serum was assayed for immunoreactivity at a concentration of 20 ng/ml by measuring binding to 5 × 108 glutaraldehyde-fixed colon carcinoma (WiDr) cells (American Type Culture Collection, Rockville, MD) overnight at RT.

Radioimmunounassay of 16.88 in Human Serum. The concentration of 16.88 in the patient’s circulation at various times postadministration was determined as follows. Immulon 1 plates with 96 wells were coated with MID 65 (10 μg/ml) in PBS and blocked with 3% fish gelatin in PBS. After a washing with 1% glycerol/0.05% Tween 20 in deionized water, either 16.88 standards (0.15 to 10 μg/ml diluted in 10% normal serum-PBS, v/v) or 10% patient serum in PBS was added to the wells together with an equal volume (50 μl) of 125I-labeled 16.88. The concentration of 125I-labeled 16.88 was determined previously by titration on MID 65 to give 80% of the maximally attainable binding level (bound cpm/total cpm). All assays were performed in duplicate. After incubation overnight at 4°C, plates were washed with PBS/1% BSA and counted for bound 125I-labeled 16.88. The standards were plotted as the fraction bound versus the amount of 16.88 added, and a line was drawn using the exponential function

\[ \ln y = \ln a + bx \]

B. Butman, personal communication.
bands (Fig. 2). Overall, MID 95 and MID 268 appeared similar to each other in reactivity, specificity, and physical properties and distinct from MID 65 in reactivity and isoelectric banding patterns.

In developing these Ab₂ mice were hyperimmunized with the human antibody plus adjuvant; over 2000 antibody-containing hybridoma cell culture supernatants were tested to obtain only four 16.88-specific Ab₂, one of which was not stable in production. It is interesting with regard to the immunogenicity of 16.88 that the three Ab₂ that could be produced reacted with identical or closely situated epitopes and, of these three, two appeared to be very similar if not identical in their reactivity and physical properties.

Application of Antidiotypic Antibodies. Because of the greater reactivity of MID 65 with 16.88 and the finding that MID 95 and MID 268 will compete with rather than complement the binding of MID 65, immunoassays to detect 16.88 in sera or tissues were developed with MID 65 alone. Human MCA 16.88 in the circulation of cancer patients being treated with this antibody was measured in a competitive binding RIA based on a “capture” format with MID 65-coated microtiter plate wells. MCA 16.88 in serum samples was quantitated by competitive binding to MID 65 with added ¹²⁵I-16.88. The dose response of 16.88 in this assay is shown in Fig. 3. The sensitivity of the method is 0.15 μg 16.88/ml patient serum. Precision within the assay measured as the SEM was within 10% at all detectable levels of 16.88 in serum. Reproducibility of individual measurements did not differ by more than 20% when assayed on different days with different preparations of ¹²⁵I-labeled 16.88. Addition of a second unrelated human MCA to the specimen did not affect the quantitation of 16.88. Fig. 4 shows the average blood clearance of 16.88 determined with sera from three patients who received a total dose of 108 mg 16.88 of which 5 mg were labeled with the radionuclide ¹²⁵I. No difference in the rate or pattern of clearance of 16.88 was apparent whether determined by counting the plasma for ¹²⁵I or by assaying the plasma for 16.88 in the Ab₂ RIA. This is an important consideration if the effect of total antibody dose on the tumor localization and pharmacokinetics is to be determined with a trace-labeled fraction. However, serum specimens collected more
These results demonstrate that MID 65 binds specifically to 16.88 and that this Ab2 can be used to detect and monitor the biodistribution of 16.88 in clinical specimens.

**DISCUSSION**

Human MCA and "humanized" murine MCA with preferential distribution to tumor tissues are being developed and studied for efficacy in the clinic. Antibodies coupled with therapeutic and diagnostic radionuclides, either directly or through a chelating agent, and antibodies coupled to nonradioactive therapeutic agents are envisioned as playing a role in the management of cancer. One part of the evaluation of a new pharmaceutical, determination of pharmacokinetics of distribution through the body and elimination by catabolism and excretion, requires accurate and sensitive methods to monitor pharmaceutical levels in the circulation, tissues, and excretory fluids. With human and "humanized" MCA quantitative assays must distinguish the pharmaceutical in an environment of greater quantities of the patient's own immunoglobulin. Specific detection relies on reactivity of the pharmaceutical with its cognate antigen or development of reagents specifically reactive with the pharmaceutical, i.e., Ab2.

Using MID 65 an Ab2 to the human MCA 16.88, we were able to monitor clearance of unlabeled 16.88 from the circulation of cancer patients and compare rates and patterns of clearance with simultaneously administered 131I-labeled 16.88. The similarity in rates of clearance indicated that the apparent clearance rate of 131I-16.88 from the blood was not increased by enzymatic removal of 131I. This finding was not unexpected; care had been taken to ensure the immunoreactivity and chemical integrity of the antibody during radiolabeling. Furthermore, dehalogenating enzymes are not notably present in the circulation. The stability of the radioiodine-labeled antibody in blood had been demonstrated prior to initiation of clinical studies. The assays served the purpose of validating the assay method for 16.88 in human serum. The value of the method will be in its usefulness in measuring levels of antibody when no tracer radioiodine conjugate can be detected as in therapy studies with nonradioactive or rapidly decaying radioactively conjugate. Intraassay precision (10%) and interassay reproducibility (20%) are within acceptable ranges for these applications.

An interesting observation was that the competitive immunoassay results showed more rapid loss of 16.88 from the circulation at 48 and 72 h postadministration than was indicated from the 131I counts/ml measurements. Also, immunoreactivity of the circulating 131I, determined by binding to cultured carcinoma cells under conditions of antigen excess, indicated a drop in immunoreactivity at the same time points. This suggests that the immunoassay was detecting only immunoreactive 131I-16.88, the difference being radiolabeled, nonimmunoreactive products of antibody catabolism or the products of its usefulness in measuring levels of antibody when no tracer radioiodine conjugate can be detected as in therapy studies with nonradioactive or rapidly decaying radioactively conjugate. Intraassay precision (10%) and interassay reproducibility (20%) are within acceptable ranges for these applications.

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<table>
<thead>
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<th>Time (days)</th>
<th>Reactivity (%)</th>
<th>Protein bound 131I (%)</th>
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<tr>
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^a Percentage of immunoreactivity of 131I-16.88 was determined as the (bound cpm/total cpm) x 100 obtained with a serum volume containing 20 mg/ml 16.88 (%10 000 cpm) reacted with 5 x 10^6 glutaraldehyde-fixed WiDr cells overnight at RT. Data are expressed as percentage of initial value (78.6 ± 13.1).
^b Determined by precipitation of protein with 10% trichloroacetic acid.
^c Mean ± SD.
Fig. 5. Autoradiographic detection of 16.88 in cryostat sections of a human colon cancer liver metastasis using $^{125}$I-MID 65. Consecutive sections of a human colon cancer liver metastasis obtained from a patient (Mo) treated with 128 mg of 16.88 eight days prior to surgery were stained with hematoxylin and eosin (left) or stained with $^{125}$I-labeled MID 65 and developed by microautoradiography (right).

Fig. 6. Autoradiographic detection of 16.88 in cryostat sections of a human colon cancer lymph node metastasis using $^{125}$I-MID 65. Consecutive sections of a human colon lymph node metastasis obtained from a patient (Hi) treated with 248 mg of 16.88 eight days prior to surgery were stained with hematoxylin and eosin (left) or stained with $^{125}$I-labeled MID 65 and developed by microautoradiography (right).
exchange of radioiodine from antibody to other circulating proteins. Additional clinical studies with higher amounts of radiolabeled 16.88 are under way. One aspect of these studies will be to determine the nature of nonimmunoreactive radiolabeled components and whether the Ab₂ used in these assays (MID 65) reacts only with immunoreactive 16.88. If correct, the immunoaassay will be additionally useful as a measurement of immunoreactivity of nonradiolabeled 16.88 antibody conjugates in the circulation.

Among tumors of similar type and structure, the antigen-binding and pharmacokinetic properties of the antibody determine patterns of localization within the tumor. Analysis of the localization patterns at the microscopic level in clinical tumor specimens has been useful with murine antitumor MCA in identifying those antibodies most applicable to antibody-targeted therapeutic applications (21, 22). Antibodies that localize in areas of healthy tumor are, presumably, better candidates than those localizing in areas of necrosis or those primarily confined to pools of mucinous secretions as have been described with anti-carcinoembryonic antigen antibodies (22). Patterns of localization may be additionally useful in designing clinical protocols for RIT and in selecting radionuclides.

Human MCA 16.88 has properties that suggest that it will be useful in RIT of solid tumors. The antibody is nontoxic at doses up to 1000 mg and nonimmunogenic even when administered as consecutive weekly doses from 8 to 200 mg for up to 5 weeks (15). Also the clinical imaging studies with ¹³¹I-labeled 16.88 indicate that the antibody is retained in tumor tissue of colon carcinoma patients for up to 23 days after administration (mean, 17 days) (15). Localization of the antibody within the tumor would provide additional valuable information regarding the use of 16.88 in RIT. The development of the monoclonal Ab₂ to 16.88 permitted such studies to be performed in human tissues without interference from endogenous human immunoglobulins. The pattern of localization of 16.88 in the tumor was strikingly tumor specific with little localization in stromal areas and little localization in areas of necrosis. The pattern was quite consistent with the requirements for effective radiomunotherapy and is encouraging with regard to further development of 16.88 in this direction.

MCA 16.88 reacts with altered forms of cytokeratins 8, 18, and 19. Cytokeratins form the intermediate filaments. Together with the smaller actin microfilaments and larger tubulin microtubules, the intermediate filaments form the cellular skeleton. Alterations in the structure of these proteins have been reported in both human (23) and murine (24) tumor cells. What may be difficult to understand is the mechanism whereby a MCA specific for cytokeratins localizes in tumor at sites of apparently healthy tumor rather than in sites of frank necrosis or distributed throughout the tumor lesion to sites of diffusion of the products of necrotic dissolution.

Dairkee and Hackett (25) addressed this question using fresh surgical specimens of breast carcinoma tissue and found that the anti-cytokeratin antibodies could be internalized by tumor cells. Normal breast epithelial cells did not take up the antibody in their studies and antibodies to stromal tissues were not internalized by the carcinoma cells. Antibody-permeable cells comprised 10 to 40% of the tumor cells in every breast carcinoma specimen assayed. The permeable cells were morphologically identical to the nonpermeable cells with no apparent signs of loss of membrane integrity or evidence of necrotic changes. It is also of interest that these authors found no localization of the anti-cytokeratin antibody in necrotic tissues which they ascribed to autolytic destruction. Likewise, in our studies, we found little localization of 16.88 in highly necrotic areas of the tumors examined. In contrast to the work of Dairkee and Hackett who exposed the tumor to antibody by immersion in a solution of antibody, tumor in our study was exposed to antibody by systemic administration 8 days prior to surgical removal. Thus, the absence of localization of antibody in necrotic areas may also reflect lack of access due to poor vascularization as it does autolytic destruction of the antigens, and both processes may be contributing.

Other investigators have found evidence of release or shedding of cytokeratins into the media of cultured tumor cells (26) as well as penetration of the tumor cell membrane by cytokeratins (27, 28). TPA has been isolated from several carcinomas and its presence in the circulation of cancer patients has been investigated extensively as a useful indicator of the presence of tumor (29). TPA is related to cytokeratins 8, 18, and 19 which, in an altered form, are recognized by 16.88 and present in most carcinoma tissues. Studies with TPA indicate that it is metabolically released from cells, possibly during cell division (29). Recently Godtfred et al. used 16.88 in in vitro studies to demonstrate that cytokeratins reactive with 16.88 are expressed as blebs on the surfaces of mammary carcinoma cells (MCF-7) but are not found on the surfaces of normal mammary epithelial cells in primary culture (24). The authors used both immunohistochemical methods and electron microscopy to demonstrate their findings.

The realization that human MCA can be developed to cytoskeletal structures of human tumor cells and that the access of antibody to these structures is cancer specific and controlled by the metabolic activity of the tumor cells is very encouraging with respect to RIT. The discovery of a new group of antigens to target in RIT is exciting in that a major limitation had been a lack of suitable antibodies that could be applied in RIT. Most clinical studies have been performed with one or more of a very small group of candidate murine MCA. A new specificity to be exploited in targeting antibodies to tumor cells presents another facet to the question of whether antibodies to cytoskeletal cell surface, secretory, or matrix components are inherently superior for RIT. An advantage to targeting carcinomas with antibodies to cytokeratins is that a relatively large amount of antigen is present per tumor cell and accessibility of antigen may allow for high density of antibody localization. Furthermore, when antigen is associated with a structure such as the cell membrane or cytoskeleton, diffusion of antibody from the tumor may be retarded to a greater extent than is expected when soluble antigen-antibody complexes are formed. This would be consistent with the long tumor residence time found with 16.88 in human colon cancer metastases (15). Finally, the prospect of treating patients with an antibody that can be given in high doses and in repeated administrations is a promising development overcoming the single most serious limitation in RIT, the inability to plan doses and dose regimens to attain maximal therapeutic efficacy.

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REFERENCES


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