In Vitro Production of the General Transformation 
Antibody Related to Survival in Human Cancer Patients: 
Antimalignin Antibody

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ABSTRACT Human antimalignin antibody (AMA) appears to have clinical significance because in actuarial studies its concentration relates quantitatively to survival (Bogoch et al. Proteids Biol Fluids 1984; 31:739-747). Therefore isolation, characterization, and production in vitro of AMA were undertaken. Serum AMA concentrations are elevated in cancer, regardless of cell type, as demonstrated by earlier blind studies of 1,026 (Bogoch et al. J. Med 1982; 13:49-69) and 501 (Bogoch and Bogoch. Proteids Biol Fluids 1983; 30:337-352) and independently confirmed by others on 354 (Bogoch et al. Proteids Biol Fluids 1984; 31:739-747) cancer patients and controls. Mouse monoclonal AMA was produced earlier (Bogoch et al. Lancet 1981; 2:141-142). To validate the identity of the natural substrate AMA in the serum determination (AMAS test) and to prepare for human imaging and therapeutic trials, human AMA has now been produced in vitro from human lymphocytes and has been shown to be increased when primed with its specific 10,000-dalton peptide antigen malignin. This synthesized human AMA adsorbs specifically to its immobilized antigen in vitro and resembles in cancer cell staining and in other properties human AMA isolated from sera of cancer patients and mouse monoclonal AMA. All are predominantly IgM, as shown by reduction to heavy and light chains followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Key words: AMA, diagnosis, monitoring, human antibody, IgM, therapy

INTRODUCTION

Antimalignin antibody (AMA) is the specific antibody to malignin. Malignin is a 10,000-dalton polypeptide whose 89 amino acids contain 13 glutamic acids and nine aspartic acids, with only two histidines, resulting in an extremely acidic molecule (pH 2.7), which requires strong acid to remove it from binding surfaces. The malignin precursor molecule, a large-molecular-weight glycoprotein designated 10B, was discovered 20 years ago [1,2] during studies on the glycoprotein structure of brain cell membranes situated at highly ordered and stable neuronal synaptic interphases. Evidence revealed that 10B appeared to be related to higher nervous system function occurring at synapses [2]. To validate the relationship of 10B

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to this stable, nondividing, mature cell state, the opposite state of rapidly dividing human brain cancer (glioblastoma) removed at surgery was examined for 10B. The 10B present in cancer cells was found to be changed in that it was increased by seven- to tenfold in its protein components but reduced in its carbohydrate components by approximately 50% [3]. The apparently amplified polypeptide subunits of this abnormal glycoprotein, termed *astrocytin* in glioblastomas in vivo and *malignin* when produced from malignant glial cells grown in tissue culture [4], were produced after several years of hydrolytic studies and were shown to be responsible for unique antigenicity in cancer. Isolated free of carbohydrate side chains, *malignin* was found to be a potent immunogen in rabbits, and its antibody (AMA) was found to be elevated in the serum of brain cancer patients [4].

During cellular hyperplasia that was not malignant, for example, in gliosis [5-7], AMA was not elevated, and malignin was not detected in cells by immunofluorescence with AMA. However, when cells underwent malignant transformation, the amount of malignin produced was directly proportional to the rate of cell division in tissue culture [4]. The cancer cell became increasingly committed to the production of the malignin polypeptide so that the percent of the cell’s total protein that could be isolated as malignin increased linearly [4]. From two lines of evidence it soon became apparent that malignin is not an antigen specific only to brain tumors. First, AMA was shown to be elevated in a wide variety of cancers, both solid and leukemic, regardless of the cell type [8-13]. Second, close structural relatives of malignin called cancer recognins were then isolated from P388 lymphoma cells (recognin L) and from MCF 7 mammary carcinoma cells (recognin M) [14]. When each of these antigens was coupled to an inert residue, such as bromoacetylcellulose, as in TARGET reagent [15], each bound AMA quantitatively in specific immunoadsorption exactly as did malignin itself, and all three antigens reacted with AMA in the same manner in Ouchterlony immunodiffusion. Malignin was therefore concluded to be a member of a group of peptides with shared structure and shared epitopes, produced by cells regardless of cell type when they become transformed, thus a general rather than a specific transformation antigen. The group was called recognins because of the loss by transformed cells of the property of contact inhibition of growth, that is, the loss of ability to “recognize” that the cells have used up the space normally available to them.

In double-blind studies of 1,026 cancer patients and controls involving nine hospitals over a 7-year period [12], it was shown that, when produced by the patient in vivo and determined quantitatively in vitro with TARGET reagent, 1) AMA was present in small quantities in healthy young normal individuals and increased only slightly with age in noncancer controls; 2) AMA was elevated in 93% of active cancer patients, regardless of cell type; AMA was elevated as early as 19 months before the clinical diagnosis was made, thus offering an aid to early detection; 3) AMA was normal in 94% of cancer cases after apparent complete eradication, as in “no further evidence of disease,” and thus was an aid to monitoring; and 4) the concentration of AMA dropped in the terminal state. Each of these four observations [12] was independently confirmed by others in a blind study of 354 cases and controls [11].

Actuarial survival analysis of a total of 515 cancer patients then demonstrated that the concentration of AMA was quantitatively related to survival in cancer patients: The more antibody, the longer the survival [11]. Thus AMA is the first general cancer antibody whose concentration has been related to survival. In addition to this relation to survival serving as an aid to prognosis and perhaps to the choice of or timing of particular therapies, the production of supplemental AMA in vitro became of interest with regard to the possibility of immunotherapy. Polyclonal AMA had been prepared in rabbits [4], human AMA had been prepared from normal and cancer sera [9], and monoclonal AMA had been prepared in the mouse [16]. These AMAs, in support of the ubiquity of the antigen malignin and the other recognins, react with single line specificity on Ouchterlony immunodiffusion and in quantitative precipitin curves against malignin. These were shown to stain malignant cells
Antimalignin Antibody Produced In Vitro

specifically (cells as diverse in type as those in histological sections from human malignant brain tumors, malignant glioma cells grown in tissue culture, human mammary carcinoma in vivo and in vitro, bronchogenic carcinoma, carcinoma of the pancreas, carcinoma of the vulva, and various lymphomas and leukemias) and to localize in vivo in scanning brain tumors in the rat at a ratio of 11:1 over normal tissue [4-7, 12-14]. To avoid the possibility of antimouse antibody reactions in patients to whom AMA might be administered, the in vitro production of human AMA was undertaken. The dual problems of synthesis of human AMA in vitro and the long-term growth of human lymphocytes had to be addressed.

MATERIALS AND METHODS

Lymphocyte Isolation and Culture

Human spleens surgically removed from noncancer (thalassemic) and Hodgkins' disease patients were minced, and lymphocytes were obtained by filtration through nylon net. These spleen lymphocytes, and both normal and Hodgkins' peripheral human B lymphoblastoid cells from heparinized blood obtained from healthy AMA serum-positive human volunteers, were collected by separation on Ficoll Hypaque gradients and sheep red blood cell rosetting. Cells were cultured in a medium consisting of RPMI 1640 containing HEPES and glutamine and 10% fetal calf serum (GIBCO, Grand Island, NY). Some cell cultures were stimulated with pokeweed mitogen (GIBCO) [17], others were transformed with Epstein-Barr (EB) virus [22], and some were unstimulated. After 7 days of cell growth, supernatants were tested for their production of AMA. The cells were then washed three times in the above medium, then ~1,000,000 cells in the medium were grown in 5-ml flasks for high-cell-density studies, and ~500,000 cells were grown in 2-ml, 24-well costar plates for low-cell-density studies. At days 2-10, cells were centrifuged at 1,000 rpm for 10 min and counted immediately, and supernatants were removed and frozen at −20°C for quantitative determination of AMA [2] within 1 week.

Quantitation of Antibody Produced

The presence of AMA in lymphocyte supernatants was defined and quantitatively determined by immunosorption to and elution from immobilized malignin as in the method for determining AMA in human serum [18]. Malignin was isolated from human malignant glial (glioblastoma) cells grown in tissue culture [4-7]. Its molecular weight by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was ~10,000 daltons [4, 14, 15]. Its amino acid composition, after hydrolysis in vacuo with 6 N HCl at 108°C for 12 hr in agreement with earlier reports [4, 14, 15], was again determined to be aspartic acid 9, threonine 5, serine 5, glutamic acid 13, proline 4, glycine 6, alanine 7, valine 6, 1/2 cysteine 1, methionine 2, isoleucine 4, leucine 8, tyrosine 3, phenylalanine 3, lysine 6, histidine 2, arginine 5. Malignin was covalently bound to bromacetylcellulose (BAC) as previously described [15] to produce immobilized malignin (BAC-malignin); 0.2 ml BAC-malignin, containing ~20 µg malignin (antigen excess), was reacted with 0.4 ml lymphocyte culture supernate at 0°C to bind AMA [18]. The BAC-malignin-AMA complex was washed with cold saline three times, incubated with 0.25 M acetic acid at 37°C for 2 hr, and centrifuged at 3,000 rpm for 20 min, and the O.D. of the clear acetic acid supernate was read at 280 nanometers and converted to micrograms antibody protein/milliliter culture supernate, as in the method described for serum [18].

Identification of Type of Immunoglobulin Produced

The acetic acid eluates of AMA, both F and S forms, were combined, neutralized, and concentrated by pervaporation to a concentration of ~200 µg/ml, reduced with dithiothreitol so that heavy chains could be examined [19], then electrophoresed on SDS-12% acrylamide gels [20].
RESULTS

Some cultures of human spleen and peripheral blood lymphocytes were found to produce (i.e., release into the culture fluid) between 5 and 10 \( \mu \text{g/mL} \) of AMA per ml culture fluid as quantitatively determined by immunoabsorption to and elution from immobilized malignin [15,18]. Addition of pokeweed mitogen (20, but not 1, \( \mu \text{g/mL} \) culture fluid) resulted in an increase in production of AMA in five of seven preparations of spleen cells from thalassemic donors to 62.0 (± 31.9) \( \mu \text{g/mL} \) and in nine of 13 preparations of Hodgkin’s spleen cells to 51.7 (± 33.3) \( \mu \text{g/mL} \), but pokeweed mitogen had no stimulatory effect on the production of AMA by peripheral lymphocytes (0/6 normal; 0/5 Hodgkin’s). This ability of splenic and peripheral blood lymphocytes to produce AMA, both unstimulated and in the presence of pokeweed mitogen, was lost within 2–4 weeks of culture; thus, neither provided a stable long-term source of human AMA.

In contrast, EB virus-transformed human peripheral B lymphocytes from normal human AMA “producers,” in both short- and long-term (6 month) culture, continuously produced AMA. Two distinct types of AMA were distinguishable in this AMA produced in culture, as previously observed in human serum [4,8,11,12] and in the two mouse monoclonal forms of AMA [15] based on differences in affinity for binding to the immobilized antigen malignin: 1) fast-binding (10 min) antibody (AMA-F) and 2) slow-binding (2 hr) antibody (AMA-S). Figure 1 shows that AMA-F production increased rapidly in the first 3 days for low-density cultures, and in the first 5 days for high-density cultures, in both instances when the cell number was increasing rapidly. However, in the case of AMA-S production, during the first 5 days of lymphocyte culture, in both high- and low-cell-density experiments, AMA-S production was minimal, only one-sixth to one-half that of AMA-F.

From approximately the sixth day, when the cell number tended to stabilize, AMA-S production increased. When the medium was renewed, or when cells were grown from liquid nitrogen, the same pattern of AMA-F and AMA-S production in relation to cell division was observed. The sequential production of AMA-F followed by AMA-S may suggest a precursor-product relationship between the two forms of AMA, but other explanations are possible and will be explored. The amount of both types of AMA produced was unrelated either to the total immunoglobulin or to the amount of IgM or IgG produced as determined by an ELISA procedure [21], which agreed with the trend of changes in AMA concentration but not with the absolute amounts. The addition to the cultures of purified malignin peptide (composition as in Materials and Methods), in concentration 0.04 \( \mu \text{g/mL} \) culture, had no effect on the cell count but was associated with an increase in the concentration of AMA-F from 5.9 (± 2.7) to 9.4 (± 4.5) \( \mu \text{g/mL} \) only in high-density cell cultures.

AMA was isolated from the supernatant fluids of lymphocyte cultures by the same immunobioabsorption method used to determine AMA quantitatively, then neutralized, and reduced to liberate the heavy chains, and these were examined by SDS-PAGE [20] (see Materials and Methods). AMA-F and AMA-S were not distinguishable by this procedure. Figure 2 (lanes 5–9) shows that, following reduction, and upon electrophoresis, the predominant heavy chains in human AMA produced in vitro are of the mu type characteristic of IgM, with molecular weight \( ~35,000 \) rather than the 55,000–65,000 for the gamma heavy chains of IgG. Unreduced AMA, as expected with a molecular weight greater than 900,000 for intact IgM, on electrophoresis remained entirely at the origin and did not enter the gel (not shown). The absence of a relationship between the amount of AMA and the total IgM produced, and that the IgM of AMA that was purified by immunobioabsorption is specific to malignin, was demonstrated by the secretion of large amounts of IgM by DAUDI lymphocytes [19] into supernates which, when absorbed on malignin, contained little or no AMA (Fig. 2, lane 4). Furthermore, Figure 3 shows that the predominant immunoglobulins of in vivo AMA isolated by immunobioabsorption from cancer patient and normal human sera (lane 3), and of mouse monoclonal AMA (lane 2), also are IgM. Immunofluorescence-activated cell
Fig. 1. Production of human antimalignin antibody in vitro. Concentration of antibody in cell culture supernatants in mg/ml supernatant. Culture density of cells, high-cell-density cultures (*) sampled on even days, low-cell-density cultures (O), sampled on odd days plus day 6. Each bar represents the mean concentration in supernate of five to seven separate cultures. In pg/ml culture = SD, of AMA-T (fast-binding), see text (solid bars) and AMA-S (slow-binding) (open bars).
Fig. 2. SDS-PAGE of in vivo and in vitro forms of antimalignin antibody. AMA was isolated from hybridoma cell culture supernates in the case of monoclonal AMA, from freshly drawn human normal and cancer serum, from Daudi lymphocyte culture supernates, and from the supernates of human lymphocyte cell cultures, each by specific immunoabsorption to malignin–BAC, as described in Materials and Methods, and reduced to show heavy chains. These heavy chains are predominantly of the mu type, 70K to 80K M.W. (arrowhead), characteristic of IgM. Lane 1: Molecular weight standards (BIORAD); lane 2: mouse monoclonal AMA; lane 3: in vivo human serum AMA; lane 4: control in vitro, Daudi lymphocytes producing high amounts of immunoglobulin but little or no AMA; lanes 5, 6: in vitro AMA produced by lymphocyte culture B. 1 month apart; lanes 7–9: in vitro AMA produced by lymphocyte culture A. 3 months apart. Light chains, ~27K M.W. (arrowhead), can also be seen.

Sorting and immunoalkaline phosphatase staining studies with human in vitro AMA on oat cell lung carcinoma cells (Fig. 3) both indicate preferential staining of cytoplasmic and outer cell membranes, consistent with that obtained with AMA isolated from human serum [5–7,12].

DISCUSSION

Human AMA made in the laboratory by human B lymphocytes is indistinguishable from the natural AMA made by the patient in all of the tests to date, such as 1) binding of the antibody specifically to its specific antigen malignin, 2) the presence of fast- and slow-reacting subtypes, 3) the predominance of the large-molecular-size immunoglobulin IgM, and 4) immunofluorescence-activated cell sorting and staining studies with human in vitro AMA on
Fig. 3. "Oat cell" lung carcinoma, grown in tissue culture, stained with human antimalignin antibody produced in vitro. Visualization using the immunoalkaline phosphatase method. Note preferential staining of cytoplasmic and outer cell membranes consistent with that previously observed with AMA isolated from human serum [4–7, 12].

carcinoma cells. The production of human AMA in vitro is important in the validation of the TARGET (AMAS) test. That is, because the AMA made in the laboratory has the same biochemical and immunological characteristics as human serum AMA both when measured quantitatively in the TARGET test and when isolated from serum, confirmation is obtained that the specific antibody being measured in the patient is the same antibody made by lymphocytes in response to the unusual peptide products of most or all cancer cells, malignin, and the related cancer recognins.

The ability to produce human AMA in vitro makes possible studies of its human cell labeling specificity, imaging, and cytotoxicity; passive immunotherapeutic trials; and the directed delivery of chemotherapeutic agents free of antimouse immunoglobulin reactions. The structural and immunological constancy of the malignin antigen over 15 years of characterization chemically in tissue culture and histologically in animals and in patients, in contrast to other antigens subject to modulation that are associated with cancer, encourages hope that its specific antibody, AMA, here produced in human form in vitro, will have a steady, nonmodulating target to label in imaging and to attack in therapeutic trials in cancer patients.

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REFERENCES