Binding and Endocytosis of a Monoclonal Antibody to a High Molecular Weight Human Milk Fat Globule Membrane-associated Antigen by Cultured MCF-7 Breast Carcinoma Cells

Esther Aboud-Pirak, Thérèse Sergent, Claire Otte-Slachmuylder, Jorge Abarca, André Trouet, and Yves-Jacques Schneider

Université Catholique de Louvain, Département de Biochimie et de Biologie Cellulaire, 74 avenue Hippocrate, B 1200 Bruxelles, Belgium

ABSTRACT

The aim of this study was to analyze whether a monoclonal antibody to human milk fat globule membrane-associated antigens, recognized specifically and homogeneously by human breast carcinoma cells but also by normal epithelial cells active in secretion, could be used to restrict the access of antitumoral drugs to cells exposing the epitope. The drug-antibody conjugate to be used is constructed by means of a covalent peptidic linkage stable in extracellular medium but hydrolyzed by lysosomal enzymes after endocytosis of the drug-carrier conjugate.

This monoclonal antibody specifically immunoprecipitates radioactive material from MCF-7 cells biosynthetically radiolabeled with galactose, glucosamine, palmitic acid, or acetic acid but not with mannose, leucine, or methionine. Upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol, the label migrates as two or three bands with apparent molecular weights of about 350,000 and 400,000. These bands disappear, or their molecular weight is affected, after treatment of the cells with cycloheximide or of cell lysates with trypsin, Pronase, and neuraminidase but not treatment of the immunoprecipitate with endoglycosidase F. This suggests that these antigens are glycoproteins with O-linked oligosaccharides containing sialic acid in the epitope. By analogy, they should be similar, if not identical, to those recognized by the monoclonal antibodies designated HMFG1 (H. Burchell, H. Durbin, and J. Taylor-Papadimitriou, J. Immunol., 131: 508-513, 1983) and DF3 (H. Sekine, T. Ohno, and D. W. Kufe, J. Immunol., 135: 3612-3615, 1985).

Binding at 4°C of the 3H-labeled antibody by MCF-7 cells indicates the specific attachment of about 1.2 x 10^4 IgG molecules per cells with a Kd of about 14 nM. At 37°C, cells take up the 3H-labeled antibody in amounts much higher than the binding capacity. This implies that cell-associated material, labeled digestion products are released into the cell culture medium. Cell fractionation by differential centrifugation and isopycnic equilibration on sucrose gradient indicates that the bulk of cell-associated antibody is distributed like the marker enzyme of lysosomes. Although the total uptake of the antibody by the cells is unaffected by either 50 μM chloroquine or 3 μg/ml cycloheximide, the release of digestion products is completely inhibited by chloroquine. Antigen-antibody dissociation is pH dependent, since, respectively, 50 and 84% of membrane-bound antibody are released during washing at pH 4.6 and 4.1.

These results support the hypothesis that once bound to a plasma membrane epitope, the antibody is rapidly endocytosed and delivered to lysosomes for digestion. Antigen-antibody dissociation could occur as a result of a lower pH in endosomes and/or lysosomes. Since the uptake at 37°C far exceeds the 4°C binding capacity of the plasma membrane and is continuous over extended periods of time, there must be a mechanism allowing the continuous supply of antigen to the plasma membrane. The absence of an effect of chloroquine, a drug known to increase the accessibility of antitumoral drugs to cells, indicates that the antibody is rapidly endocytosed and delivered to lysosomes.

All these results strongly suggest that this antibody is endocytosed, gains access to lysosomes, and could therefore be an appropriate carrier for drug targeting based on the lysosomotropic concept.

INTRODUCTION

An approach to restrict the access of antitumoral drugs to cancer cells consists of associating them with macromolecular carriers recognizing with high affinity and selectivity binding sites at the surface of target cells (1). In an attempt to demonstrate this concept, a covalent linkage has been constructed between drugs and proteins in a way ensuring stability during transport of the drug-carrier conjugate in extracellular fluids but liberation of the drug in an active form within the cells (2). In such an approach, drug release relies on the endocytosis of the drug-carrier conjugate, on its access to lysosomes, and on the susceptibility of the linkage between the drug and the carrier to lysosomal enzymes.

This approach was validated in the case of the treatment of the exoerythrocytic form of murine malaria, in which the parasites reside within the hepatocytes. A conjugate was constructed by linking primaquine, a drug active against this form of the disease, through a succinylated tetrapeptide arm to asialofetuin, a glycoprotein specifically recognized and endocytosed by hepatocytes and then delivered to lysosomes. When tested on mice infected with Plasmodium berghei, the conjugate was found to be more active and less toxic than the free drug (3).

The specific aim of this work was to study whether monoclonal antibodies raised to epitopes exposed at the surface of human tumor cells could be used for targeting anticancer drugs according to this conceptual approach. As an experimental model, we have selected human breast tumor cells and antigens which, although not tumor specific, could allow partial selectivity. HMFGM were chosen as a possible example. Biochemical and morphological evidence indicates that HMFGM derive from the apical membrane of cells active in milk secretion (4-7). Antibodies to HMFGM were found to be specific for most normal epithelial cells active in secretion as well as for tumoral deriving cells. Among the numerous MAbs to HMFGM or to breast carcinoma cells, several recognize a family of high molecular weight tumor-associated glycoproteins comprising two distinct molecules in MCF-7 cells (8), a human metastatic cultured breast carcinoma line. On the other hand, the transferrin receptor could be of particular concern, since it is present

Received: 6/19/87; revised 12/15/87; accepted 2/22/88.

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.

1. This work was supported by the Belgian Fonds de la Recherche Scientifique Médicale (Grant 3.4568.84).

2. Present address: The Weizmann Institute of Sciences, Department of Chemical Immunology, P. O. B. 26 Rehovot, 76100 Israel.

3. To whom requests for reprints should be addressed.

The abbreviations are: DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; HMFGM, human milk fat globule membrane-associated antigen; MAAb, monoclonal antibody; PBS, phosphate-buffered saline (NaCl, 140 mM; KCl, 3 mM; NaHPO4-KH2PO4, 10 mM; pH 7.4); SDS, sodium dodecyl sulfate; MLP, particulate fraction made up of heavy and light mitochondrial and microsomal fractions.
at the cell surface of most, if not all, tumor cells. Furthermore, the transferrin receptor rapidly enters the cell, gains access to endosomes wherein transferrin iron is released, and is recycled back to the cell surface allowing detachment of iron-depleted transferrin (9).

In this paper, we report on the molecular characterization of the antigen recognized by a MAb to HMFGM which recognizes homogeneously breast carcinoma cells (10). The antigen was biosynthetically radiolabeled in MCF-7 cells with different precursors and then immunoprecipitated under various conditions. We also describe the interaction of this MAb with MCF-7 cells. The binding, uptake, and subcellular localization of the 3H-labeled antibody has been studied, as well as the effect of chloroquine and cycloheximide. Results indicate that this antibody could be used to target antitumor drugs to MCF-7 cells according to our model, since it binds to plasma membrane, is endocytosed, and gains access to lysosomes. In a forthcoming paper, we will present data on the cytotoxic activity of conjugates constructed with this antibody and daunorubicin or vinorelbine.

MATERIALS AND METHODS

Preparation of HMFGM. HMFGM were prepared from milk samples obtained from mothers who volunteered at the local hospital. Milk was diluted twice with 0.25 M sucrose in 10 mM Tris HCl, pH 7.5-7.1 M MgCl2 (11). The cream was separated by centrifugation for 15 min at 3000 x g at room temperature and washed three times with buffer. The suspension was first kept at 4°C for 3-4 h and allowed to reach room temperature. Membranes were released from fat globules by churning (VirTis Gardiner, New York, NY) and separated by centrifugation at 150,000 x g for 1 h (L5 50 centrifuge, T50 rotor; Beckman, Palo Alto, CA). Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as standard. Typically, about 3 mg of protein were obtained from 100 ml of milk.

Preparation and Labeling of MAb. Female BALB/c mice were given s.c. injections of 50 µg of HMFGM protein in Freund's complete adjuvant (Difco Laboratories, Detroit, MI); at 4-week intervals, mice were given injections of the same amount of antigen in complete adjuvant, first i.p. and then s.c. Six months later, but 3 days before sacrifice, 5 mg of antigen without adjuvant were given i.v.

Spleen cells from immunized mice, collected in DMEM (Gibco-BRL, Ghent, Belgium), were fused with SP 2/0 mouse myeloma cells grown in DMEM containing 10% (v/v) FCS (Gibco-BRL, Ghent, Belgium), were fused with SP 2/0 mouse myeloma cells grown in DMEM containing 10% (v/v) FCS (Gibco-BRL) at a ratio of 5:1:5-10% (v/v) polyethylene glycol 1000 (Sigma Chemical Co., St. Louis, MO) in serum-free DMEM at pH 7.4. Cells were then plated (150 x 105 cells/well) in 96-well microplates (Nunc, Roskilde, Denmark) in DMEM containing 10% FCS and supplemented with 100 µM hypoxanthine, 380 nM aminopterin, and 16 µM thymidine.

The secretion of specific antibody by hybridoma was assayed on 96-well microplates (Nunc) coated overnight at 4°C with 10 µg/ml of HMFGM, extemporaneously sonicated, in 40 mM glycine buffer, pH 9.2, containing 30 mM NaCl. After washings of unbound antigen with 10 mM Tris, pH 8.0, containing 0.05% (v/v) Tween 20 (Sigma), plates were incubated with 50 µl/well of hybridoma supernatants for 90 min at 37°C. After washings, 50 µl/well of horseradish peroxidase sheep F(ab')2 to mouse immunoglobulin (NEN, Boston, MA) diluted 1:500 in 100 mM Tris, pH 8.0, containing 0.05% (v/v) H2O2, in 17 mM citrate buffer, pH 6.5, were added in the dark. Reaction was stopped with 4.5 mM H2SO4 and reaction products were detected photometrically at 495 nm.

 Supernatants from hybridoma reactive with HMFGM were further assayed with MCF-7 cells seeded in 96-well microplates in DMEM containing 10% FCS and fixed with 4% (v/v) formaldehyde in PBS. The assay was performed as above except that washings were carried out with 1% (w/v) bovine serum albumin in DMEM.

Selected hybridoma were cloned by limiting dilution techniques and then injected i.p. (2 x 106 cells in DMEM containing 1% FCS/mouse) into female BALB/c mice, primed 1 month before with 0.5 ml pristane (Sigma). Antibodies were purified from the ascitic fluids by affinity chromatography on Sepharose-protein A (Pharmacia, Brussels, Belgium). The antibody eluted with 0.1 M citrate, pH 6.0. Determination of specific antibody binding with specific antibodies indicates that the MAb belongs to the IgG1 subclass. Antibodies were labeled with Na2H3 (Amersham, Brussels, Belgium) by reductive methylation of lysines using a method adapted from Ref. 13. Typically, a specific radioactivity of about 500 dpm/ng of IgG was obtained.

Anti-transferrin receptor MAb was secreted by the OKT9 hybridoma (ATCC, Rockville, MD). Control MAb of the IgG1 subclass was kindly provided by Dr. J. Van Snick (Université Catholique de Louvain, Unité de Médecine Experimentale). The absence of cross-reactivity of this MAb with HMFGM and MCF-7 cells was checked as described above.

Cell Culture and Biosynthetic Labeling. MCF-7 cells, deriving from a malignant pleural effusion in a female patient with breast carcinoma and kindly provided by Dr. Mercier-Bodard (Université de Paris-Sud, France), were routinely cultured in DMEM supplemented with 10% (v/v) FCS and 40 IU/liter of insulin (Gibco, BRL). Viability of the cells was routinely controlled at the beginning and the end of the experiment by phase contrast microscopy. Cells do not detach and the monolayer remains intact.

For biosynthetic labeling, cells were incubated in 25-cm2 flasks (Becton-Dickinson, Oxnard, CA) for 18 h at 37°C with 1.2 ml of complete culture medium supplemented with 10 µCi/L[35S]methionine, 10 µCi/L[3H]-leucine, 10 µCi/L[3H]glucosamine, 10 µCi/L[3H]glactoside, 10 µCi/L[3H]mannosone, 10 µCi/L[3H]palmitic acid, or 10 µCi/L[3H]acetic acid, all purchased from Amersham.

Immunoprecipitation. After biosynthetic labeling, the medium was removed and the cells were washed twice with PBS and solubilized for 30 min on ice in 1% (v/v) Nonidet P-40 and 1% (v/v) sodium deoxycholate in 10 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml of bovine serum albumin.

The cell lysate was centrifuged for 5 min at 2,000 x g (IEC DPR 600, rotor 250; IEC, Needham Heights, MA) and the supernatant was further centrifuged at 4°C for 30 min at 100,000 x g (Beckman L5 50, T50). The lysate was preclarified by 1 h incubation at 4°C with rabbit serum followed by 30 min incubation with 50 µl/ml of a 10% (w/v) suspension of fixed Staphylococcus aureus (Calbiochem, San Diego, CA) in lysis buffer. Bacteria were removed by centrifugation (12,000 x g for 10 min) in a Beckman microfuge. The supernatant was incubated with 10 µg/ml of the MAb for 2 h at 4°C, followed by 1 h of incubation at 4°C with 50 µg/ml of rabbit anti-mouse immunoglobulin (Dako, Glandrup, Denmark) and 1 h at 4°C with 50 µg/ml of S. aureus. The precipitated immune complexes were washed three times with 0.5% (v/v) sodium deoxycholate-1% (v/v) Triton X-100 in 142 mM NaCl-240 mM KC1-8 mM NaH2PO4-1.4 mM K2HPO4. pH 7.2.

After washings, the immunoprecipitates were dissociated by boiling for 5 min in 2% (w/v) SDS-10% (w/v) glycerol-1% (w/v) diethiothreitol-0.005% (w/v) bromophenol blue-80 mM Tris-HCI, pH 6.8. Electrophoretic separation was performed by the method of Laemmli (14) in 4-15% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed by soaking in 30 min in methanol-acetic acid-water (45/49/6, v/v), treated with Amplify (Amer sham), and dried. Autoradiography was carried out at ~70°C with Kodak X-Omat AR5 film.

Enzymatic Treatments. For some experiments, the cell lysate was digested twice with either trypsin (0.25% w/v) in PBS, pH 7.4, containing 3 mM EDTA, with 7000 units/ml Pronase (Calbiochem) in PBS, or with 1 unit/ml neumainidase (Sigma) in 0.1 mM sodium acetate buffer, pH 5.5, containing 0.3 mM NaCl and 0.2% (w/v) CaCl2. Incubation with enzymes was carried out for 2 h at 37°C. The mixture containing neuraminidase was then cooled on ice and the pH was adjusted to 7.4 prior to immunoprecipitation.

For some other experiments, after immunoprecipitation the antigen was released by boiling for 5 min in SDS-dithiothreitol-glycyl-Tris.
After centrifugation, the supernatant was diluted with 100 μl of 50 mM EDTA-1 mM phenylmethylsulfonyl fluoride-0.5% (v/v) Nonidet P-40 in 0.1 M sodium phosphate buffer, pH 6.1, containing 10 units/ml of endoglycosidase F (Boehringer Pharma, Brussels, Belgium). Enzymatic digestion was performed for 17 h at 37°C and terminated by adding 10 μl of 10% (v/v) SDS-10% (w/v) glycerol-1% (w/v) dithiothreitol in 0.1 M Tris-HCl buffer, pH 6.8, and boiling for 5 min.

**Binding and Uptake Experiments.** For binding and uptake experiments, cells were grown to confluency in 25-cm² flasks. Cells were then incubated in 2 ml of complete culture medium with the H²-labeled MAb. At the end of the incubation, the culture medium was removed and kept for analysis while the cells were washed twice with 2 ml of culture medium and three times with 2 ml of PBS either at 4°C or at room temperature. The cells were then dissolved in 1 ml of 1% (w/v) sodium deoxycholate adjusted to pH 11.3 with NaOH and assayed for protein (12) with bovine serum albumin as standard and for associated radioactivity after dispersion of the samples in Aqualuna cocktail (Lumac Systems, Basle, Switzerland) in a Tri-Carb 460 CD scintillation counter (Packard Instruments, San Diego, CA) with automatic corrections for sample quenching. For experiments at 37°C, the culture media were further analyzed for antibody digestion by measuring the amount of ³H label soluble in 15% (w/v) trichloroacetic acid after precipitation and centrifugation of protein. As control, culture medium containing ³H-labeled antibody was incubated in parallel at 37°C but in the absence of cells.

To estimate the protein content of MCF-7 cells, two confluent 175-cm² flasks were cultured in parallel; one was used for the determination of protein content as described above, whereas cells from the other were detached with trypsin and counted in a Bucker cell. Results indicate that 1 mg of cell protein corresponds to 3.0 ± 0.8 (SD) × 10⁶ cells.

**pH Dependence.** To estimate the dissociation of H²-labeled MAb bound to MCF-7 cells as a function of pH, confluent cultures in 25-cm² flasks were incubated for 1 h at 4°C in the presence of H²-labeled MAb. Cells were then washed at 4°C once with chilled DMEM containing 10% FCS and three times with chilled PBS, and then reincubated for 10 min at 4°C with 10 mM citrate buffers of different pH values in 0.15 M NaCl. After washings with PBS, cell-associated radioactivity and protein were assayed as above.

**Experiments with Drugs.** To assay leucine incorporation into material precipitated by 15% (w/v) trichloroacetic acid, confluent MCF-7 cells were incubated with ³H]leucine (0.8 μCi; 50 Ci/mmol) in the presence or absence of cycloheximide at different concentrations.

Confluent MCF-7 cells were incubated for 24 h at 37°C with 30 μg/ml of H²-labeled antibody in the presence or absence of 50 μM chloroquine. After washings as above, cells were harvested; samples containing 12.5 μg of cell protein were treated and analyzed by polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol as above.

**Subcellular Localization.** Confluent MCF-7 cells in two 175-cm² flasks were incubated with 15 ml of culture medium containing the H²-labeled MAb. Cells were then washed as above and harvested with a rubber policeman in 0.25 M sucrose-3 mM imidazole, pH 7.0. The suspension was pelleted for 3 min at 350 × g (IEC DPR 6000, rotor 259). Cells were homogenized by 6 cycles of 2 strokes of the tight pestle of a Dounce homogenizer followed by a 10-min centrifugation at 700 × g at 4°C to separate the nuclear fraction (N). Pooled supernatants of a Dounce homogenizer were washed twice with DMEM supplemented with 10% (v/v) and coverslips in Petri dishes were incubated at 4°C or 37°C with culture medium containing control or specific MAb. After incubation, cells were washed twice with DMEM supplemented with 10% (v/v) and three times with PBS and then fixed with 4% (v/v) formaldehyde in PBS for 5 min at room temperature. To permeabilize membranes, cells were incubated for 4 min at room temperature in the presence of 0.1% (v/v) Triton X-100 in PBS and washed three times with PBS. Permeabilized cells were then reincubated for 3 h at room temperature in the presence of peroxidase-conjugated rabbit antibody to mouse immunoglobulin (Dako) diluted 40 times in PBS. Diaminobenzidine (Sigma) and H₂O₂ were used as substrates for peroxidase (18). The slides were counterstained with hematoxylin, dehydrated, and mounted before examination.

**RESULTS**

**Selection of Hybridoma.** The fusion of myeloma cells (SP 2/0 line) with splenic lymphocytes from mice immunized with HMFGM resulted in a large number of hybridoma. They were first screened for secretion of antibody reactive with HMFGM on a solid phase enzyme-linked immunosorbent assay positive clones were further selected for immunoreactivity to epitopes exposed at the cell surface of cultured MCF-7 cells. After subcloning, some of the cell lines keeping immunoreactivity for MCF-7 cells and growing well in culture after freezing and thawing and in ascites were chosen; after purification of the corresponding MAb, a preliminary characterization of their reactivity with normal and tumoral tissues and cells by immunohistochemistry (10) and, after labeling, of their binding and uptake by cultured MCF-7 cells (19) was performed. One MAb deriving from the hybridoma named 7F11C7 and characterized as an IgG1 was selected for further studies and possible use as drug carrier.

**Characterization of the Antigen.** In a first set of experiments, MCF-7 cells were biosynthetically radiolabeled with different precursors. Cell lysates were treated without mouse antibody or with either the specific MAb or, as a control, with a MAb to the transferrin receptor (OKT9). The immunoprecipitates were separated by polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol and then analyzed by autoradiography. Whereas no label appears on the gel in the absence of treatment of the lysate with a mouse antibody, after immunoprecipitation with the specific MAb two bands corresponding to material with apparent molecular weights of about 350,000 and 400,000 are present after labeling of the cells with radioactive galactose, glucosamine, palmitate, and acetate (Fig. 1), but not with mannose, leucine, and methionine (not illustrated). When immunoprecipitation was carried out with the OKT9 MAb, a single band with an apparent molecular weight of 90,000 was observed with all the precursors.

In order to further characterize the nature of these antigens, different treatments were performed. Results, illustrated at Fig. 2, indicate that after incubation of cell lysates with Pronase or neuraminidase followed by the immunoprecipitation with the specific MAb no labeled band appears on the gel. After the cell lysate was treated with trypsin, the molecular weight of the antigen is decreased; no effect was observed after incubation of the immunoprecipitate with endoglycosidase F. In contrast, the immunoprecipitation of the transferrin receptor is sensitive to treatment with trypsin, Pronase, and endoglycosidase F, but not with neuraminidase (not illustrated).

If the biosynthetic radiolabeling of MCF-7 cells is performed in the presence of 10 μg/ml cycloheximide, which inhibits [³H]-leucine incorporation into labeled material precipitable by trichloroacetic acid to more than 95%, no labeled band appears on the gel after immunoprecipitation with either the specific and the OKT9 MAb.

**Low Temperature Binding.** Binding of the MAb to the plasma membrane of MCF-7 cells was performed at 4°C in order to minimize endocytosis. In a first set of experiments,
Fig. 1. Autoradiography of antigens radiolabeled biosynthetically in MCF-7 cells. Subconfluent cultures of MCF-7 cells (about 10^6 cells in 25-cm² flasks) were incubated for 18 h at 37°C with 50 μCi/ml of [3H]glucosamine (Lanes 2 and 3), [3H]mannose (Lanes 4 and 5), [3H]acetic acid (Lanes 6 and 7), [3H]palmitic acid (Lanes 8 and 9) or [3H]galactose (Lanes 10 and 11). Lysis of the cells, immunoprecipitation with the specific (Lanes 2, 4, 6, 8, and 10) or the OKT9 (Lanes 3, 5, 7, 9, and 11) MAbs, polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol, as well as autoradiography were carried out as described in "Materials and Methods." MC-labeled molecular weight standards (Lane 1) were: myosin, 200,000; phosphorylase b, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000.

Fig. 2. Effect of different treatments on the molecular weight of the antigens. MCF-7 cells were biosynthetically radiolabeled with [3H]glucosamine. Cell lysates, as such (Lane 2), or after treatments with trypsin (Lane 3), Pronase (Lane 4), or neuraminidase (Lane 5) were immunoprecipitated with the specific MAb. Immunoprecipitate from untreated cells was also incubated in the presence of endoglycosidase F (Lane 6). Other protocols were as in Fig. 1. Lane 1, 14C-labeled standards as in Fig. 1.

Fig. 3. Low temperature binding of the MAbs to MCF-7 cells. In A, confluent cultures of MCF-7 cells (about 2 x 10^6 cells in 25-cm² flasks) were incubated for 3 h at 4°C with 2.5 ml of DMEM containing 10% (v/v) FCS and supplemented with different concentrations of [3H]-labeled specific (•) or control (○) MAb. At the end of the experiment, cells were washed and analyzed as described in "Materials and Methods." Values are mean results of three independent experiments ± SD (bars). B, Scatchard plot of the binding of the specific MAb illustrated in A. B, number of moles of IgG bound per mg of cell protein; F, molar concentration of free IgG in the culture medium. K_a and average number of IgG molecules per cell were estimated by attributing 3 x 10^6 MCF-7 cells to 1 mg of protein and considering a 1/1 stoichiometry to the antigen-antibody reaction.

Fig. 4. Time dependence of the uptake of specific and control MAbs by the cells. Confluent cultures of MCF-7 cells in 25-cm² flasks were incubated at 37°C for different durations with 2 ml of DMEM containing 10% (v/v) FCS supplemented with 10 μg/ml of [3H]-labeled specific or control MAb. At the end of the experiment the amount of labeled digestion products in the culture medium was determined after precipitation of protein by 15% (w/v) trichloroacetic acid, whereas washed cells were assayed for radioactivity and protein content. Experimental procedures are described in "Materials and Methods." Values are mean results of three independent experiments ± SD (bars). ○, cell-associated labeled material; △, labeled digestion products in the culture medium; •, total uptake (△ + ○) of the specific MAb; □, total uptake of the control MAb.

control MAb increases proportionally to its extracellular concentration. At an extracellular IgG concentration of 10 μg/ml the binding of the specific MAb is 19 times higher than that of the control MAb. Considering a 1/1 stoichiometry of the antibody-antigen reaction and 3 x 10^6 MCF-7 cells per mg of cell protein, the Scatchard analysis (21) of these data indicates a homogeneous population of high affinity (K_a ~ 14 nM) binding sites with an average of 1.2 x 10^6 sites per cell (Fig. 3B).

When MCF-7 cells were incubated for 3 h at 4°C with 10 μg/ml of [3H]-labeled specific MAb in the presence of a 100-fold excess of the unlabeled MAb, the binding of [3H] label is inhibited by 93%.

Uptake of the Antibody. The time dependence of the uptake of the [3H]-labeled specific and control MAbs by MCF-7 cells is illustrated in Fig. 4. For the specific MAb, the amount of cell-associated [3H] label increases as a function of the incubation period for 6 h and then proceeds more slowly. After a lag phase
of about 6 h, \(^{3}H\)-labeled digestion products soluble in trichloroacetic acid appear in the culture medium in concentrations increasing proportionally to the duration of the incubation. Summing up the amount of cell-associated label (i.e., accumulation) and of labeled digestion products in the culture medium, the resulting uptake increases up to at least 48 h; at the end of 24 h, the accumulation level corresponds to about \(7.0 \times 10^{6}\) antibody molecules per cell and the digestion to about \(2.4 \times 10^{6}\) molecules. For the control MAbs, the amounts of cell-associated \(^{3}H\)-labeled material are much lower and no labeled degradation products can be detected in the culture medium.

As indicated in Table 1, in the presence of 50 \(\mu\)M chloroquine, a drug known to increase lysosomal (22) and endosomal (23) pH and to affect endocytosis and lysosomal digestion (24), the release of labeled digestion products soluble in trichloroacetic acid is completely inhibited; concomitantly, the amounts of cell-associated label increases resulting therefore in unsignificantly affected total uptake. Polycarylamide gel electrophoresis in the presence of SDS and dithiothreitol indicates that in both the presence and the absence of chloroquine, cells accumulate molecules with apparent molecular weights of about 25,000 and 50,000, suggesting intact light and heavy immunoglobulin chains (not shown).

Cycloheximide, at 3 \(\mu\)g/ml in the extracellular medium which inhibits to more than 90\% \(^{3}H\)-leucine incorporation into material precipitable by trichloroacetic acid (not illustrated), has no effect on the accumulation of labeled MAb or on the release of labeled digestion products in the culture medium (Table 2).

**Washout of the MAb.** When MCF-7 cells, preincubated for 3 h at 37°C with 10 \(\mu\)g/ml of the \(^{3}H\)-labeled specific MAb are washed and reincubated in antibody-free culture medium, the amount of cell-associated \(^{3}H\) label decreases progressively and, after 24 h, reaches 20\% of the initial accumulation level; most of the labeled material released in the culture medium consists of digestion products soluble in trichloroacetic acid (Fig. 5).

In order to assay the reappearance of antigen molecules at the cell surface, MCF-7 cells were first incubated for 16 h at 37°C with 30 \(\mu\)g/ml of unlabeled MAb. Cells were then washed and reincubated at 37°C in the absence of MAb. After different durations, cells were washed again and reincubated for 3 h at 4°C with 30 \(\mu\)g/ml of \(^{3}H\)-labeled MAb. In the absence of washout in fresh medium at 37°C, the binding of \(^{3}H\) label was

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Accumulation ((\mu)g/mg cell protein)</th>
<th>Digestion ((\mu)g/mg cell protein)</th>
<th>Uptake ((\mu)g/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 50 (\mu)M chloroquine</td>
<td>3.30 ± 0.08</td>
<td>2.95 ± 0.05</td>
<td>6.25 ± 0.13</td>
</tr>
<tr>
<td>+ 50 (\mu)M chloroquine</td>
<td>5.91 ± 0.09</td>
<td></td>
<td>5.91 ± 0.09</td>
</tr>
</tbody>
</table>

**Table 2. Effect of cycloheximide on the uptake of the antibody**

Confluent cultures of MCF-7 cells were incubated for 24 h at 37°C with 2 ml of DMEM containing 10\% (v/v) FCS and supplemented with 30 \(\mu\)g/ml of the \(^{3}H\)-labeled MAb. After washings with PBS, cells were reincubated for 10 min at 4°C with 2 ml of 0.14 M NaCl containing 10 mM citrate buffers of different pH. Results are expressed as percentage of cell-associated \(^{3}H\) label at the end of washout over that remaining bound to the cells reincubated at pH 7.4. Values are mean results of three independent experiments.

**Table 1. Effect of chloroquine on the uptake of the antibody**

Confluent cultures of MCF-7 cells in 25-cm\(^2\) flasks were preincubated for 1 h at 4°C with 2 ml of DMEM containing 10\% (v/v) FCS and supplemented with 10 \(\mu\)g/ml of the \(^{3}H\)-labeled MAb. Values are mean results of three independent experiments ± SD.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Duration of incubation (h)</th>
<th>Uptake ((\mu)g/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cycloheximide</td>
<td>6</td>
<td>2.46 ± 0.15</td>
</tr>
<tr>
<td>+ Cycloheximide</td>
<td>24</td>
<td>4.78 ± 0.10</td>
</tr>
</tbody>
</table>

**Fig. 5. Washout of the specific MAb from the cells.**

Confluent cultures of MCF-7 cells in 25-cm\(^2\) flasks preincubated for 3 h at 37°C with 2 ml of DMEM containing 10\% (v/v) FCS and supplemented with 10 \(\mu\)g/ml of the \(^{3}H\)-labeled MAb, washed three times with 2 ml of PBS, were reincubated for different durations at 37°C with 2 ml of the same medium in the absence of antibody. Cells and medium were analyzed as in Fig. 4. Values are mean results of three independent experiments.

**Fig. 6. pH dependence of the dissociation of the specific MAb.**

Confluent cultures of MCF-7 cells in 25-cm\(^2\) flasks were preincubated for 1 h at 4°C with 2 ml of DMEM containing 10\% (v/v) FCS and supplemented with 10 \(\mu\)g/ml of the \(^{3}H\)-labeled MAb. After washings with PBS, cells were reincubated for 10 min at 4°C with 2 ml of 0.14 M NaCl containing 10 mM citrate buffers of different pH. Values are mean results of three independent experiments.

**Fig. 6. pH dependence of the dissociation of the specific MAb.**

Confluent cultures of MCF-7 cells in 25-cm\(^2\) flasks were preincubated for 1 h at 4°C with 2 ml of DMEM containing 10\% (v/v) FCS and supplemented with 10 \(\mu\)g/ml of the \(^{3}H\)-labeled MAb. After washings with PBS, cells were reincubated for 10 min at 4°C with 2 ml of 0.14 M NaCl containing 10 mM citrate buffers of different pH. Values are mean results of three independent experiments.
ENDOCYTOSIS OF A MONOCLONAL ANTIBODY BY MCF-7 CELLS

Fig. 7. Cell fractionation by differential centrifugation. Confluent cultures of MCF-7 cells (about 30 x 10^6 cells in 175-cm^2 flasks) were incubated for 24 h at 37°C with 10 ml of DMEM containing 10% (v/v) FCS and supplemented with 10 µg/ml of specific or control 3H-labeled MAb. After washings, cells were harvested; homogenized; separated into N, MLP, and S fractions; and analyzed as described in "Materials and Methods." Ordinate, relative specific activity of each constituent in a given subcellular fraction; abscissa, percentage of protein present in that fraction in a cumulative form from left (N fraction) to right (S fraction).

Fig. 8. Cell fractionation by isopycnic centrifugation. The particulate (MLP) fractions from the experiments described in Fig. 7 were further separated on a linear sucrose gradient. Fractions were separated and analyzed and the results are expressed as described previously (16, 17).

37°C with 3H-labeled specific or control MAb. In both cases the bulk of cathepsin B and 5'-nucleotidase activities are associated with the MLP fraction; respectively, 53% (specific) and 91% (control) of the 3H label are found in this fraction. For the specific antibody, label is also recovered in the S fraction and 47% of it consists of labeled material precipitable by trichloroacetic acid, suggesting that it consists of intact IgG released from organelles during homogenization or fractionation (not illustrated).

Isopycnic centrifugation of the MLP fraction on a linear sucrose gradient (Fig. 8) reveals that in the case of both the specific and the control MAb, the 3H label equilibrates around median densities of 1.17 g/ml overlapping to a large extent the distribution of cathepsin B, the marker enzyme of lysosomes. For the specific antibody, a shoulder is, however, observed towards lower densities where 5'-nucleotidase, the marker enzyme of plasma membrane and related organelles, is detected.

Nonsignificantly different results are obtained when the cells were incubated in the presence of 3H-labeled F(ab) fragments of the MAb (not illustrated).

Morphological Examination. Fig. 9 illustrates the interaction of the specific and control MAb with MCF-7 cells. After incubation of the cells with the specific MAb at 4°C, permeabilization of cellular membranes, incubation with a second antibody to mouse IgG, and cytochemical reaction, the staining is mainly found at the plasma membrane, lining the boundaries between cells (Fig. 9A). After incubation with the specific MAb at 37°C, although some immunological reaction is still located on the plasma membrane, most of the staining is clearly located in perinuclear granules within the cytoplasm, suggesting that part of the antibody has been endocytosed (Fig. 9, B and D). No staining was observed after incubation at 4°C with a control MAb (Fig. 9C).

DISCUSSION

Using the hybridization technology, a panel of MABs to HMFGM has been obtained. Among those reactive with epitopes displayed at the cell surface of MCF-7 cells, one, named 7F11C7, reacts homogeneously with all tumor cells present in breast primary carcinomas as well as with deriving metastases, but also with many different other normal epithelia active in secretion and deriving tumoral tissues (10). This MAb appears to have an immunological profile comparable to others the properties of which have been reported in the literature and suggested to recognize breast tumors from other cells in culture (5, 26), for tumor detection by immunohistochemical methods (6, 7, 11), for tumor prognosis (27) and, in vivo, for tumor imaging in patients (28, 29).

Preliminary results (10) have indicated that although not tumor specific, this antibody could be used to target antitumor drugs by restricting their delivery to cells exposing the epitope at the pole which is accessible from blood or extracellular fluid. Nevertheless, experiments were designed, on one hand, to characterize the antigen recognized by this MAb and, on the other hand, to determine more precisely whether this MAb fulfills the criteria required for an appropriate transport relying on a covalent linkage constructed between the drug and the carrier and on a lysosomal release of the drug after endocytosis of the conjugate (1–3).

This MAb, after biosynthetic radiolabeling of MCF-7 cells, specifically immunoprecipitates two molecules with apparent molecular weights of above 350,000 and 400,000 upon polyacrylamide gel electrophoresis in the presence of SDS and dithiothreitol. These molecules are distinct from the transferrin receptor, used as control, and immunoprecipitated by the OKT9 MAb (30).

The sensitivity of the antigens recognized by the specific MAb to treatment of cell lysate with trypsin or Pronase and to incubation of the cells with cycloheximide suggests that they have a protein content, although they do not incorporate detectable levels of leucine and methionine. The absence of effect of endoglucosidase F, which hydrolyzes high mannosé and complex oligosaccharidic structures (31), suggests that they are glycoproteins with a saccharidic moiety that incorporate galac-
Endocytosis of a Monoclonal Antibody by MCF-7 Cells

Fig. 9. Indirect immunocytochemical localization of the MAbs. Nonconfluent cultures of MCF-7 cells on coverslips in 20-cm² Petri dishes were incubated for 90 min at 4°C (a, c) or for 24 h at 37°C (b, d) with DMEM containing 10% (v/v) FCS and supplemented with 10 μg/ml of the specific antibody (a, b, d) or of the control MAb (c). Washings, fixation, and permeabilization of the cells as well as the indirect immunoperoxidase reaction were carried out as described in "Materials and Methods." a, b, c, × 570; d, × 780.

Tose and glucosamine but not significantly mannose and that are not linked to asparagine. The effect of neuraminidase suggests that the epitope recognized by the MAb should contain sialic acid.

Altogether, these biochemical properties strongly support the hypothesis that these antigens are similar, if not identical, to those recognized by the MAb designated, respectively, HMFG1 (32) and DF3 (33).

Binding experiments as well as morphological examination indicate that, at 4°C, the MAb is recognized with high affinity and specificity by antigens exposed at the plasma membrane of MCF-7 cells. At 37°C, the antibody is endocytosed by MCF-7 cells and thereafter processed within lysosomes: (a) morphological examination (Fig. 9) of cells incubated for 24 h with the antibody before membrane permeabilization and reincubation with anti-mouse IgG antibody reveals that most of the immunocytochemical staining is localized within perinuclear granules, which, by analogy to previous work (34, 35), are lysosomes; (b) cell fractionation by differential (Fig. 7) and isopycnic (Fig. 8) centrifugation indicates that the bulk of cell-associated 3H label distributes like the marker enzyme of lysosomes, although some material behaves like plasma membrane marker or is found in cytosol, probably as a result of release from lysosomes after digestion or from damaged organelles during homogenization or fractionation; (c) continuous uptake (Fig. 4) or wash-out experiments (Fig. 5) demonstrate the release into the culture medium of labeled digestion products soluble in trichloroacetic acid in a process which is entirely blocked by chloroquine (Table 1). The fact that antibody molecules are stored within lysosomes as material which migrates upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol as intact light and heavy immunoglobulin chains could appear contradictory. We have, however, described previously that rabbit polyclonal IgG resists for a rather long duration to lysosomal enzymes before being entirely broken down (36).

A model can be tentatively proposed to explain these results. After binding to antigenic molecules displayed at the plasma membrane, the MAb would be endocytosed. Considering, on the one hand, the pH prevailing in endosomes, i.e., around pH 5.0 (24, 25) and, on the other hand, the partial dissociation of the antigen-antibody complex occurring at this pH (Fig. 6), part of the antibody molecules could dissociate from their epitopes. This would be comparable to what has been reported for several ligands after receptor-mediated endocytosis (37–40).

By analogy, the antibody molecules could then travel within vesicles from endosomes to lysosomes, to be digested, while antigen molecules would be recycled back to the plasma membrane.

3194
Nevertheless, since the endosomal pH does not seem to be low enough to promote complete antigen-antibody release, two hypotheses could be considered: undissociated complexes could either be recycled back to cell surface and cycle until dissociation takes place at random; or, possibly as a result of cross-linking of two antigenic molecules by each IgG molecule, cross-linked epitopes could be transported to lysosomes. However, the similar subcellular distributions observed upon isopycnic centrifugation of fractions obtained from cells incubated with either the entire MAb or its F(ab) fragment argue against this hypothesis.

Within lysosomes, antigen-antibody could dissociate under the action of the more acidic pH and/or the effect of lysosomal enzymes. Antibody molecules could thereafter stay within lysosomes and be progressively degraded with release of low molecular weight labeled products outside the cells while antigen molecules could either be digested or escape digestion and be recycled back to cell surface.

The comparison of the number of antibody molecules bound to the plasma membrane at 4°C (i.e., about 1.2 × 10^6 molecules/cell) with the amount captured during 24 h incubation at 37°C (i.e., about 9.4 × 10^6 molecules/cell) clearly indicates that the uptake capacity of the cells far exceeds the binding capacity of the plasma membrane. Moreover, kinetic experiments further confirm that the uptake of the MAb by the MCF-7 cells proceeds continuously, even though not proportionally to the duration of the incubation, over at least 48 h. This process must therefore result from a continuous supply at the cell surface of epitope molecules able to bind the antibody.

These antigens appearing at the cell surface could theoretically originate from, at least, three distinct origins. They could first result from the recycling of epitopes that have escaped to lysosomal digestion after a round of endocytosis, either from endocytic vesicles or from lysosomes. This could be compared to what happens to several receptors such as e.g., those for asialoglycoproteins (37), low density lipoprotein (41), transferrin (42), and many others or to antigens to which polyclonal antibodies were bound, as we have described previously (35). However, the observation that chloroquine has no effect on the uptake of the antibody by the cells, although this drug is known to increase endosomal and lysosomal pH and should therefore inhibit the intracellular dissociation of the antigen-antibody complex, does not seem to support the hypothesis that recycling of endocytosed antigens would be a limiting step for the continuous uptake of antibody molecules.

Secondly, they could derive from the neosynthesis of antigen molecules. The absence of any effect of cycloheximide, a potent protein synthesis inhibitor, does not seem to support this hypothesis, especially since our results suggest that the antigen is a glycoprotein, the synthesis of which should be affected by this drug.

Finally, antigen molecules appearing at the cell surface could find their origin in an intracellular pool of already existing epitopes. This pool should, however, be large enough to supply the plasma membrane over extended periods of time. This hypothesis could be in agreement with our previous morphological results indicating the presence of intensive cyttoplasmic staining in both breast tumor specimens obtained from patients and in MCF-7 cells (10). It seems further supported by the observation that, after preincubation of MCF-7 cells with the MAB, antigens reappear at the cell surface, even in the presence of cycloheximide.

Taken together, these data strongly suggest that this MAB fulfills the criteria required to be used as a drug carrier according to the lysosomotropic concept (43, 44). Obviously, the next step is therefore to test this approach in an experimental model after constructing a conjugate between this antibody and antitumor drugs.

ACKNOWLEDGMENTS

The excellent technical assistance of C. Decamps-Michel and M. Wauters is gratefully acknowledged. Typing of the manuscript was carefully performed by M. Marchand and R. Cottet.

REFERENCES


Binding and Endocytosis of a Monoclonal Antibody to a High Molecular Weight Human Milk Fat Globule Membrane-associated Antigen by Cultured MCF-7 Breast Carcinoma Cells

Esther Aboud-Pirak, Thérèse Sergent, Claire Otte-Slachmuylder, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/11/3188