New Chimeric Anti-Pancarcinoma Monoclonal Antibody with Superior Cytotoxicity-mediating Potency

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ABSTRACT

The monoclonal antibodies (MAbs) 323/A3 and 17-1A both recognize a 40-kDa carcinoma-associated epithelial glycoprotein (EGP40). MAb 17-1A has been used in many therapeutic trials as an immunotherapeutic agent to combat colon and pancreatic cancer, and about 5-10% overall response have been observed. It has been shown that MAb 323/A3 has a higher affinity than 17-1A, which might be an advantageous feature for a therapeutic agent. In our immunohistological studies different reaction patterns of these two MAbs were observed, suggesting that MAb 323/A3 might be a more effective immunotherapeutic tool. Because chimerization may reduce the immunogenicity of the murine MAb 323/A3 and increase the interaction with human effector mechanisms, we developed a chimeric form of murine MAb 323/A3. MAb 323/A3 heavy and light chain variable genes were cloned and grafted onto human C-v and C-y domains, respectively. A chimeric antibody-producing cell line was established by transfection of the chimeric constructs into a nonproducing myeloma cell line. The chimeric and murine 323/A3 MAbs were evaluated for efficacy of inducing complement-mediated cytotoxicity (CMC) and mediating antibody-dependent cellular cytotoxicity against LS 180 cells derived from human colon carcinoma. Both forms were found to mediate similar levels of CMC in the presence of human complement; however, higher levels of lysis of target cells were observed with human peripheral blood lymphocytes when the chimeric 323/A3 was used. Chimeric 323/A3 mediated higher maximal cytotoxicity than chimeric 17-1A in both CMC and antibody-dependent cellular cytotoxicity assays and was equally active as chimeric 17-1A at 100-1000-fold lower concentrations. The superior reactivity of chimeric 323/A3 with EGP40 on carcinoma cells and its higher cytotoxicity-mediating capacity, compared to chimeric 17-1A, are important characteristics, which support further clinical studies with chimeric MAB 323/A3 in immunotherapy of carcinomas.

INTRODUCTION

In recent years MAbs 1 to tumor-associated antigens have become an important treatment modality in the immunotherapy of cancer (reviewed in Refs. 1-3). The efficacy of MAbs in therapy, however, appears to be influenced by many factors, including affinity and the number of binding sites on the target cell, antigen expression on tumor versus normal tissue, and vascularization of the tumor. Immunotherapy, especially with low-affinity antibodies, requires injection of relatively high doses of MAb, which leads in most patients to the development of an immune response against the injected murine MAb (1) with possible numerous side effects. The use of chimeric MAbs with high affinity to an antigen, which is preferentially expressed on tumor cells, might improve the efficacy of these potentially powerful tools.

The human EGP40, encoded by the GA733-2 gene (4), is expressed at high levels on the surface of the majority of carcinomas, including breast, ovary, colon, and lung cancers (5-7). Colon carcinoma cells express approximately $1 \times 10^6$ EGP40 molecules per cell (1). Because of its wide occurrence and high expression level, EGP40 is an attractive target for the immunotherapeutic treatment of carcinoma patients (1). MAb 17-1A, reactive with this pancarcinoma antigen (8), has been extensively studied in clinical trials as an immunotherapeutic tool to combat colon (2) and pancreatic (9) carcinomas. Some complete remissions were observed at the stage of advanced disease (10). The modest efficacy of MAb 17-1A in these clinical trials might be related to its low affinity, leading to high injected doses (up to 12 g) (1, 11) needed to achieve therapeutic effects. These high doses of murine antibody resulted in the development of a HAMA response in almost all patients treated with this MAb (1). In order to reduce the HAMA responses and to increase the interaction with human Fc-yRI high-affinity receptors as well as with complement, a c17-1A was generated by grafting the murine variable immunoglobulin genes onto human constant immunoglobulin genes (12). This chimerization increased the plasma half-life of the monocular antibody (13) and diminished HAMA responses. However, the relatively high doses needed in patients makes MAb 17-1A less attractive for use in immunotherapy of advanced disease.

Another EGP40-reactive MAb 323/A3 (14) exhibits an affinity of $2 \times 10^4$ M$^{-1}$, which is approximately 40-fold higher than the affinity of MAb 17-1A (15). In the present study these two antibodies were compared for their tumor reactivity on sections of carcinomas of different origins. MAb 323/A3 appeared to react with a significantly higher percentage of tumor cells in a wide variety of adenocarcinomas and squamous cell carcinomas than did MAb 17-1A. The superior tumor recognition of MAb 323/A3 compared to MAb 17-1A suggests a wider applicability of MAb 323/A3 in immunotherapeutic treatment of different carcinomas, whereas its higher affinity might lead to improved efficacy. Based on these data and the observation that chimerization improves the MAb interaction with human effector mechanisms (16), we developed a human-mouse chimeric form of MAb 323/A3 by replacing the murine antibody constant domains (C-v and C-y) with the human C-v and C-y, respectively. In the present study we show that chimeric MAb 323/A3 mediates superior cytotoxicity against human colon carcinoma cells in vitro, compared to murine MAb 323/A3 as well as to chimeric and murine MAb 17-1A.

MATERIALS AND METHODS

Cell Lines. The 323/A3 hybridoma, P3X63Ag8.653 murine myeloma cells (American Type Culture Collection CRL 1580), and transfectants of the latter were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (HyClone, Logan, UT). Tumor cell line LS 180 (colon adenocarcinoma, American Type Culture Collection CL 187) was grown in DMEM with 10% fetal calf serum (GIBCO, Paisley, United Kingdom). All cells were grown at 37°C and 5% CO$_2$. 

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Monoclonal Antibodies. The murine MAbs 323/A3 (14) and 17–1A (8) were both of the IgG2a isotype. MAB 323/A3 IgG2a, originally an IgG1 isotype, was generated by subcloning and selection. The chimeric forms of 323/A3 and 17–1A both have human IgG1 constant domains (12). Controls in ADCC and CMC were the murine IgG1 isotype of 323/A3 and MAB 139H2, a murine IgG2a anti-breast carcinoma antibody (17), and the chimeric IgG1 version of 7E3 (anti-platelet GPlb/IIla) described in Ref. 18. Muring IgG1 SF25 and chimeric SF25 (anti-hematopoïa) (19) were used as nonbinding controls in the competition assays. All antibodies used were purified over a protein A-Sepharose (Pharma Biotech B.V., Woerden, the Netherlands) column, and after further purification by sequential ion-exchange chromatography on Mono Q and Mono S (Pharmacia) columns they were dialyzed into PBS. All MAB preparations used were negative for endotoxin activity.

Immunohistochemistry. Immunohistochemistry was carried out on frozen tissue sections, obtained from the tumor bank of the Department of Pathology, Leiden, as described before (20). To summarize the data into an overall result, the average percentage of cells positive with 323/A3 or Vh variable domains was calculated. For each tumor type the percentage of positive tumor cells per individual tumor was determined. The percentage of positive tumor cells per group was calculated by summarizing the percentages of positive tumor cells per individual tumor and dividing by the total number of tumors tested per group. Sections from normal tissues expressing the EGP40 antigen served as positive controls. The data were statistically evaluated using an unpaired t test.

DNA Probes. The mouse variable chain probes were previously described by Looney et al. (21). The Jh probe contains a 2.0-kilobase BamHI/EcoRI fragment of the murine J3 and J4 heavy chain exons. The mouse light chain Jk probe is a 2.7-kilobase HindIII fragment containing all five murine Lk exons. Probes were labeled with 5'-<32P]dCTP (Amersham, Buckinghamshire, United Kingdom) using a random priming kit (Boehringer Mannheim, Indianapolis, IN). A Sephadex G-50 spin column was used to separate the labeled probe from unincorporated nucleotides. The specific activity of the probes was approximately 1 X 10^6 cpm/μg DNA.

Isolation of Genomic and cDNA of MAB 323/A3 Vh and Vk Regions. cDNA clones containing the 323/A3 variable region domains were obtained as described by Caten et al. (22) and Kavalier et al. (23). Briefly, specific primers for the constant heavy and light chain domains of the MAB were used to generate by PCR the first strand of cDNA from total RNA. Second-strand cDNA was obtained by amplification of the G-tailed first cDNA strand using a primer that generated the first strand of cDNA from total RNA. Second-strand purified and used at a concentration of 0.1 μg/100 μl in a standard 100-μl PCR plasmid DNA preparations (24). K. cherichia HB101 was used for all plasmid constructions and primers using a Sequenase dideoxy sequencing kit (United States Biochemical Corp., Cleveland, OH). Genomic DNA isolated from the 323/A3 hybridoma was digested with EcoRI and separated on a 0.7% agarose gel. The DNA was subsequently transferred to a nitrocellulose filter (Schleicher & Schuell, Keene, NH) and hybridized with the radiolabeled Jh probe. A 3.0-kilobase fragment was found to be homologous with the Jh probe, and sections from normal tissues expressing the EGP40 antigen served as positive controls. The data were statistically evaluated using an unpaired t test.

Expression Vectors. The expression vectors pHukapger and pH1agart containing the human constant κ- and γ1-coding genes, respectively, and the xanthine-guanine phosphoribosylase gene (gpt) have been described by Looney et al. (21).

Generation of Chimeric Antibody-producing Cell Line. The heavy and light chain expression vectors containing the genomic DNA fragments that encode the murine 323/A3 Vh or Vk variable domains were purified on a DNA isolation column (Qiagen, Chatsworth, CA) and linearized with BarnHI. Each plasmid (20 μg) was combined and introduced into 2 X 10^7 nonproducing mouse P3X63Ag8.653 myeloma cells by electroporation. Transfected clones were selected in medium containing 0.25 μg/ml mycophenolic acid, 1.25 μg/ml hypoxanthine, and 25 μg/ml xanthine. The surviving clones were examined for antibody production by ELISA.

Identification of Chimeric Antibody-producing Cells by ELISA. Production of chimeric antibody by the selected clones was assayed by a standard ELISA technique using plates coated with 1 μg/well goat anti-human IgG Fe antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Subsequently, 50 μl culture supernatant were added per well and detected with alkaline phosphatase-conjugated goat anti-human IgG (H+L) antibody in Tris-buffered saline and 1 μg/ml Sigma 104 phosphatase substrate in alkaline buffer solution (Sigma, St. Louis, MO). The reaction was stopped by adding 50 μl 3 N NaOH/well and examined at 405 nm on an ELISA plate reader (Dynatech, Chatsilly, VA). Antibody concentrations were calculated from a standard curve generated from a standard purified chimeric antibody (γ1,k).

Competition Assay. Murine 323/A3 was labeled with 125I using the iodogen method (25). A series of 12 subsequent 1:2 dilutions of the cold antibody (starting at 720 μg/ml), mixed with 1 μg 125I-labeled murine MAB 323/A3 in 50 μl, was added to 1 X 10^5 LS 180 cells in 50 μl PBS, 1% bovine serum albumin/well in a 96-well V-bottom plate. After 18 h incubation at 4°C and centrifugation at 1000 rpm for 10 min, the supernatants were removed. The pellets were washed twice with PBS containing 1% bovine serum albumin and counted for 125I in a gamma counter.

Complement-mediated Cytotoxicity Assay. A CMC assay was performed using 1 X 10^6 LS 180 colon carcinoma cells, labeled with 100 μCi 51Cr in normal saline (Amersham) for 90 min at 37°C and 5% CO2. The cells were washed twice with 2 ml DMEM and resuspended at a concentration of 2 X 10^6 cells/ml. DMEM (50 μl) containing MAB (yielding final concentrations between 100 and 0.001 μg/ml in 1:10 dilutions) was added to 100 μl 51Cr-labeled LS 180 cells in 96-well round-bottom microtiter plates (Greiner, Langenthal, Switzerland). Subsequently, 100 μl of each batch of human AB+ serum (pooled from healthy donors) was added as a complement source to obtain final serum concentrations of 30, 10, 2, and 0.5%. After the plates were incubated for 4 h at 37°C and 5% CO2, 100 μl supernatant were removed and counted (ER, experimental release) in a LKB gamma counter. The maximum release (MR) was obtained by adding 100 μl 1% Triton X-100 to 100 μl labeled cells plus 50 μl medium. Spontaneous release (SR) was obtained by incubating 100 μl labeled LS 180 cells with 150 μl medium. The percentage specific lysis was calculated as

\[
\text{Specific Lysis} = \frac{\text{ER} - \text{SR} - \text{MR} - \text{SR}}{\text{MR}} \times 100
\]

Antibody-dependent Cellular Cytotoxicity Assay. ADCC assays were performed using LS 180 cells as targets and, in all experiments, human peripheral blood lymphocytes from the same healthy donor. The effectors were from 30 to 50 units/ml interleukin-2 (Eurocetus, Amsterdam, the Netherlands) as effectors. LS 180 cells (1 X 10^6) were labeled with 100 μCi 51Cr (Amersham) at 37°C and 5% CO2. Cells were washed twice with 2 ml DMEM and resuspended at a concentration of 1 X 10^6 cells/ml. DMEM (50 μl) containing MAB was added to 100 μl LS 180 cells (1000 cells/well) in 96-well round-bottom microtiter plates (Greiner), and subsequently, effector cells were added at E:T ratios of 100, 50, 25, 12, and 6:1. After the plates were incubated 3 h at 37°C, 100 μl supernatant were removed and counted. The percentage specific 51Cr release was calculated as described for the CMC. All CMC and ADCC data were statistically analyzed using an unpaired t test.

Antibody-binding Assay. LS 180 cells (5 X 10^5) were incubated for 1 h at 4 or 37°C with MAB in 1:10 dilutions (starting at a concentration of 10 μg/ml) in 2 ml PBS. Cells were subsequently washed with PBS and incubated for 1 h at 4°C with 100 μl fluorescein isothiocyanate-labeled anti-human IgG (Southern Biotechnology Association, Birmingham, AL), 1:100 diluted in PBS. After the cells were washed with PBS they were resuspended in 300 μl

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RESULTS

Reactivity of MAbs 323/A3 and 17–1A with Different Carcinomas. The reactivity of murine MAbs 323/A3 and 17–1A was compared on frozen sections of various human carcinomas of different grades. MAb 323/A3 showed clear cell membrane staining in all carcinomas tested, in contrast to 17–1A, which notably stained only gastric and colon carcinomas (Fig. 1). The percentage of tumor cells recognized was significantly higher (P < 0.01) in all tumor groups for MAb 323/A3 than for MAb 17–1A. Additionally, staining of tumors with MAB 323/A3 was much more intense than the staining with MAb 17–1A, except in gastric and colon carcinoma, in which the intensities were similar (data not shown).

Cloning of the MAb 323/A3 Variable Genes. We cloned MAb 323/A3 variable genes to produce a chimeric 323/A3 MAb. Clones containing the putative 323/A3 heavy and light chain variable region genes were isolated from the phage genomic libraries by hybridization with the radiolabeled JH or JK probes, respectively. Reverse transcriptase PCR was used to amplify heavy and light variable regions from total RNA isolated from the 323/A3 hybridoma. The nucleotide sequence of the heavy and light variable chain cDNA was determined (not shown) and used to design primers specific for the CDR3 regions of the 323/A3 variable regions. These primers were used with a universal 5′ primer to amplify the CDR3 regions of the heavy and light genomic variable genes from the selected phage clones by PCR. Clones that were positive in this PCR were sequenced, and, with exception of the intron sequences, the genomic DNA matched the cDNA sequences of the variable 323/A3 domains. The variable heavy chain sequence showed homology to previously described VH regions (26) and contained a sequence coding for a 19-amino acid leader and an open reading frame coding for the heavy chain variable region joined to the JH2 region. The complete nucleotide sequence of the 323/A3 VH region encoded for a 20-amino acid leader and a 339-base pair open reading frame coding for the κ-chain variable region joined to the Jκ1 region (not shown).

Sequence comparison of 323/A3 VH with the variable heavy chain domains of MAbs 17–1A and GA733–2, which also recognize EGP40, revealed that, unlike the striking homology between 17–1A VH and GA733–2 VH (27), 323/A3 uses a totally different variable heavy chain gene.

Expression of the Chimeric 323/A3 Antibody. The light and heavy chain variable region genes cloned from the 323/A3 hybridoma were joined to human Cκ and γ1 constant region genes, respectively, in expression vectors previously described (21). After transfection, 52% of the clones selected produced the 323/A3 chimeric antibody, as determined by ELISA. Culture supernatants from the transfectants were tested for the ability to inhibit binding of radiolabeled murine MAB 323/A3 to LS 180 cells (Fig. 2). This assay confirmed that the antibody produced by the transfectants competed with binding of the murine MAB 323/A3. The clones that produced the highest amounts of antibody (30–50 μg/ml) were subcloned. A clone producing 70 μg/ml MAB c323/A3 was identified, and antibody from the culture supernatant of this clone was purified and used in further experiments.

Complement-mediated Cytotoxicity by MAbs 323/A3 and 17–1A. The ability of the murine and chimeric versions of the 323/A3 and 17–1A antibodies to mediate lysis of EGP40-expressing LS 180 human colon carcinoma cells in the presence of human complement was tested. Chimeric 323/A3 mediated an approximately 2-fold higher maximal CMC than MAB c17–1A at an antibody concentration of 100 μg/ml (Fig. 3). At 0.1 μg/ml c323/A3 still gave the maximal cytotoxicity, whereas c17–1A was not able to mediate CMC at 10 μg/ml (Fig. 3). The murine 323/A3 IgG1 and the isotype-matched control murine IgG2a (139H2) or chimeric IgG1 MAbs (c7E3) could not induce CMC at any MAb or serum concentration tested (data not shown). To compare the interaction of human complement with either human or mouse constant immunoglobulin regions, CMC was performed with the murine IgG2a and chimeric IgG1 versions of both the 323/A3 and 17–1A MAbs. Chimeric 323/A3 and c17–1A mediated similar CMC as their murine IgG2a counterparts at all MAb concentrations tested (Fig. 3). Between 100 and 0.1 μg/ml, both chimeric and murine 323/A3 mediated significantly higher CMC (P < 0.01) than chimeric and murine 17–1A. Mab c17–1A lost its ability to induce CMC at concentrations <10 μg/ml, whereas c323/A3 maintained maximal activity until an antibody concentration of 0.1 μg/ml. A threshold phenomenon could be observed because CMC was abrogated completely within a 10-fold dilution of both 323/A3 and 17–1A. This would be expected if a minimal number of antibody molecules had to

PBS containing 1 μg/ml propidium iodide to discriminate between viable and dead cells. From each sample 10,000 viable cells were examined for the amount of MAb bound.
be bound to its epitope to induce CMC. The described difference in CMC between both 323/A3 and 17-1A chimeric and murine versions was observed at human serum concentrations varying between 30 and 2% (data not shown).

**Antibody-dependent Cellular Cytotoxicity with MAbs 323/A3 and 17-1A.** The ability of c323/A3 and c17-1A to mediate lysis of LS 180 cells by human PBLs was tested. Both c323/A3 and c17-1A mediated maximal amounts of cellular lysis at an antibody concentration of 10 μg/ml. However, at antibody concentrations of 0.1 and 0.01 μg/ml, the ability of MAb c17-1A to mediate ADCC decreased, while MAb c323/A3 maintained the ability to mediate almost maximal cellular lysis. Even at the lowest concentration tested, 0.001 μg/ml, c323/A3 still mediated substantial lytic activity (Fig. 4). ADCC with human PBLs, derived from the same batch, and the chimeric and murine versions of MAbs 323/A3 and 17-1A on LS 180 cells revealed that c323/A3 mediates significantly better ADCC (P < 0.01) than all other MAbs tested. Both chimeric 323/A3 and 17-1A MAbs consistently mediated higher ADCC than their murine IgG2a counterparts (Fig. 4). No ADCC activity over background levels was observed using murine 323/A3 IgG1 or a nonreactive murine IgG2a or chimeric MAb. These results show that MAb c323/A3 mediated ADCC comparable to that mediated by c17-1A at a 100- to 1000-fold lower concentration (Fig. 4). The ADCC results were achieved using an E:T ratio of 25:1; however, at E:T ratios of 100:1, 50:1, 12.1, and 6:1, similar results were obtained with MAb c323/A3, mediating significantly higher ADCC (data not shown).

**Relative Binding of c323/A3 and c17-1A to LS 180 Cells.** The difference in the ability of c323/A3 and c17-1A to mediate cytolysis might be due to the total amount of antibody bound to the target cells. Therefore, we determined the binding of different concentrations of c17-1A and c323/A3 to LS 180 cells at 37 and 4°C using FACS analysis (Fig. 5). The antibody to cell ratios in the binding assay were the same as used in ADCC and CMC assays. The FACS analysis data showed that both chimeric 323/A3 and 17-1A MAbs bind to LS 180 cells. The amount of MAb 323/A3 bound to the cells at 10 and 1 μg/ml was approximately 2-fold higher than the total amount of 17-1A bound. At lower concentrations, binding of 323/A3 was 4- to 10-fold higher than binding of 17-1A. The difference in binding was observed after 1 h between the incubations at 37 and 4°C. The decrease in amount of antibody bound at 37°C at high concentrations was not observed when cells were incubated with antibody at 4°C. However, the differences in total amount of MAbs 17-1A and 323/A3 bound to the cells were not affected. Binding of murine MAbs 323/A3 and 17-1A did not differ from the binding of the chimeric versions (data not shown). Therefore, the lack of CMC observed with 17-1A at 0.1 μg/ml cannot be solely due to antibody binding, since at a concentration of 0.01 μg/ml c323/A3 binding to LS 180 cells was only slightly elevated over c17-1A but gave more than half-maximal CMC (Fig. 3). Binding of c17-1A at 0.1 μg/ml is also clearly higher than binding of c323/A3 at 0.001 μg/ml. Yet, the latter concentration of c323/A3 mediates a significantly higher level of ADCC than c17-1A at 0.1 μg/ml (Fig. 4).

**DISCUSSION**

In the present study, we describe the generation of a chimeric MAb 323/A3, recognizing EGP40, by molecular cloning of the heavy and light chain variable 323/A3-coding genes and joining them to the human constant K- and 7-domains, respectively (reviewed in Refs. 28 and 29). EGP40 (4, 30) is expressed by most cells of a majority of human tumor tissues as reported in this study. This pancarcinoma antigen is therefore an attractive target for immunotherapy. MAb affinity has been shown, using theoretical and mathematical models, to influence MAb uptake into the tumor and thus affect therapeutic results. The models predict that it is important to have a reasonably high-affinity MAb (K<sub>D</sub> > 10<sup>-8</sup> M<sup>-1</sup>; 32, 33). Shockley et al. (34) state that, based on their model, one would not expect an

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**Fig. 3. CMC on LS 180 human colon carcinoma cells, presented as the mean percentage 51Cr (column) released in 4 h, mediated by chimeric 323/A3 (□) and 17-1A (○) IgG1 and murine 323/A3 (□) and 17-1A (○) IgG2a at a human serum concentration of 33%, which served as source of complement.** This is representative of four different CMC experiments on LS 180 cells with chimeric and murine antibodies at different serum concentrations carried out in triplicate on 1 day. Nonbinding control chimeric MAb 7E3 (IgG1) and murine MAb 139H2 (IgG2a) mediated no lysis of LS 180 cells. Murine 323/A3 IgG1 did not mediate CMC as well. MAb c323/A3 gave significantly higher lysis (P < 0.01) of LS 180 cells than did chimeric and murine MAb 17-1A (*). At 0.001 and 0.1 μg/ml c323/A3 mediated significantly higher lysis than did c17-1A, m323/A3 (P < 0.05) and m17-1A (**). Bars, SE.

**Fig. 4. Antibody-dependent cellular cytotoxicity of 51Cr-labeled LS 180 human colon carcinoma cells mediated by chimeric 323/A3 (□) and 17-1A (○) and murine versions of 323/A3 (□) and 17-1A (○) MAbs and human PBLs during a 4-h period.** Percentage (column) of specific 51Cr release is plotted against a titration of the MAb concentration (E:T ratio of 25:1). This is a representative of five different ADCC experiments on LS 180 cells at different E:T ratios (100, 50, 25, 12, and 6:1) carried out in triplicate on 1 day with the same human PBL batch. Isotype-matched nonbinding control murine MAb 139H2 (IgG2a) and chimeric MAb 7E3 (IgG1) did not give 51Cr release over background. Chimeric 323/A3 gave significantly higher lysis of LS 180 human colon carcinoma cells (P < 0.01) than did the other MAbs (*) at all MAb concentrations tested in ADCC. Bars, SE.
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Fig. 5. Binding of chimeric forms of MAb 323/A3 (•) and MAb 17–1A (○) to LS 180 human colon carcinoma cells as measured by FACS at 37°C (A) and 4°C (B). Mean fluorescence channel number of each sample was plotted against c323/A3 and c17–1A antibody concentration used for incubation. Antibody concentration and amounts versus cell number ratio were adjusted to those used in ADCC and CMC assays.

additional effect on tumor uptake of increasing MAb affinity beyond $10^9 \text{ M}^{-1}$ (34). Such arguments would favor the use of the high-affinity ($K_a = 2 \times 10^9 \text{ M}^{-1}$) MAb 323/A3 (14, 15) over the low-affinity MAb 17–1A ($K_a = 5 \times 10^7 \text{ M}^{-1}$) (8, 15). However, other factors, such as tumor epitope accessibility, vascularization of the tumor, antigen density (32, 35), and tumor size (36, 37), may influence antibody localization into the tumor as well. Therapeutic efficacy may also be dependent on more subtle interactions of the antibody with the effector mechanisms.

In addition to the expected positive influence of MAb affinity and chimerization on MAb uptake by the tumor, we show here that MAb c323/A3 is superior to c17–1A in mediating cytolytic activity as measured by CMC and ADCC, especially at low concentrations. Since the amount of antibody bound will be related to the ADCC and CMC effects, a binding study was performed on LS 180 cells at 37°C for 1 h. Optimal binding was seen for c323/A3 and c17–1A at 0.1 and 1 µg/ml, respectively. At 4°C, however, an optimum was not detected, suggesting that the observed decrease in amounts of bound antibody at 37°C at high MAb concentrations was due to internalization. At concentrations below the optimas seen at 37°C, the antibody binding was similar at both temperatures. There are two possible explanations for the fact that internalization of antibody was not reflected in the ADCC results: (a) the cells may be lysed before the internalization starts, which is not likely since we have observed a linear relationship between incubation time and cytotoxicity (data not shown), and (b) even when internalization takes place the target cells may still express amounts of antibody on their surface capable of sustaining plateau levels of ADCC. Differential binding of MAbs c17–1A and c323/A3 to tumor cells as analyzed by FACS cannot completely explain the different levels of cytotoxicity mediated by c323/A3 and c17–1A. For CMC we observed a threshold phenomenon as reported before by Wong and Colvin (38). Even when a significant amount of antibody was bound to LS 180 cells as detected by FACS at 37°C, the CMC activity decreased to background for both c323/A3 and c17–1A, suggesting that a minimal number of bound antibody molecules are required to induce CMC. Although with 17–1A at 37°C and 0.1 µg/ml approximately 3-fold more MAb is bound to LS 180 than with 323/A3 at a concentration of 0.001 µg/ml, this amount of bound 323/A3 is able to exert approximately 30% more ADCC than 17–1A. Therefore, the higher efficiency of MAb 323/A3 compared to 17–1A in mediating ADCC is clearly not due to higher affinity only. The discrepancy observed between antibody bound to target cells and level of ADCC observed, in combination with the difference between MAb 17–1A and MAb 323/A3 in staining intensity and reaction pattern on frozen tissue sections of various tumors, strongly suggests that these two MAbs recognize different epitopes on the EGP40 molecule. Sequence comparison of GA733 and 17–1A with the 323/A3 variable heavy chain supports this assumption. MAbs 17–1A and GA733 both recognize EGP40 and compete for binding to this molecule with MAb 323/A3 (39). The amino acid sequences of the heavy chain variable regions of 17–1A and GA733 have been shown to be similar, and their CDR3 amino acid sequences are identical (27). These data strongly suggest that these two MAbs recognize the same epitope. However, the 323/A3 heavy chain variable region including its CDR3 amino acid sequence is completely different, suggesting that a different epitope on EGP40 is recognized, which negates the hypothesis by Caton (27), that the murine antibody response against EGP40 produces structurally related antibodies that recognize the same epitope. Competition of 323/A3 with 17–1A for EGP40 does not exclude the recognition of different epitopes by these antibodies but could be related to steric hindrance or affinity. Pak et al. (40) also suggested that the epitopes for MAbs 323/A3 and 17–1A are not the same but may be partly overlapping.

Mediation of ADCC via the antibody Fc regions is dependent on the distance between Fc tails, and this distance may be related to the epitopes recognized. This statement is strengthened by the observation that dimeric forms of IgG give better ADCC results in vitro (41) and increase tumor destruction in vivo (42). If MAbs 323/A3 and 17–1A indeed recognize different epitopes on EGP40, the discrepancy in ADCC and total amounts of 17–1A and 323/A3 antibody bound may be explained by assuming that binding of 323/A3 may create better targets for complement and Fc receptors. The different staining patterns observed for 323/A3 and 17–1A on various types of carcinomas may, apart from affinity differences, be related to differences in accessibility of epitopes recognized or to the expression in some tumors of a different molecular form of EGP40 (e.g., other glycosylation) that is not recognized by MAb 17–1A in contrast to MAb 323/A3. Different epitopes on the same antigen recognized by MAbs have previously been shown to affect ADCC by human effector cells (43).
The difference observed in ADCC between the murine and chimeric versions of both MAbs 323/A3 and 17-1A cannot be due to epitope recognition because this does not change upon chimerization. Several chimeric isoforms have been tested in the past, and the chimeric IgG1 form was found to be superior in ADCC and CMC over other chimeric isoforms (44). The data about differences in ADCC performance of chimeric MAbs versus their murine counterparts are rather conflicting. Although most reports show an improved ADCC for chimeric IgG1 versus murine IgG2a (16, 45, 46), some groups find hardly any differences between versions (47). This might be explained by different relative levels of FcγRI, II, and III on the effector cell populations (44, 48, 49) or by the significant variation in ADCC potential of PBLs observed between donors (50). Additionally, different interactions of chimeric and murine MAbs with the human FcγRI could be invoked as the explanation for differences in ADCC. Despite the presence of an identical FcγRII-binding sequence on murine IgG2a and human IgG1 (51), it is likely that conformational differences in the Fc parts make the human IgG1 provide a more efficient triggering signal for the human FcγRI than murine IgG2a. Finally, our results confirm the findings of others that IgG2a is the most effective murine isotype for CMC and ADCC mediation in the human system, since we found the murine 323/A3 IgG1 totally ineffective in these assays (44).

It has recently been stated by Langmuir et al. (15) that for tumor penetration a MAb with low affinity (17-1A) is preferable over a MAb with high affinity (323/A3). They found that MAbs with high affinity will preferentially saturate antigenic sites on the tumor cells on the border of the tumor loci as well as around blood vessels (35). However, the impact of reduced penetration into a tumor nodule may be no problem when therapy with MAbs can be extended. Observations that the effector cells for ADCC as well as complement factors are mainly located on the borders of tumor loci (20, 52) are in favor of the use of high-affinity MAb c323/A3. Moreover, to combat micrometastases, poor penetration of high-affinity antibodies would not be an issue.

The results presented in this paper show the superiority of chimeric MAB 323/A3 in in vitro tumor cell lysis. The low concentration at which c323/A3 can perform optimal cytolytic activity compared to MAB 17-1A may be important for clinical application. Both MAbs 17-1A and 323/A3 react on tissue sections with malignant and normal epithelium. However, binding of the MAbs to normal epithelium will be low in vivo because of poor diffusion of antibodies through the basal membrane and gap junctions. Therefore, MAB 323/A3, despite its higher affinity, is not expected to cause increased damage to normal epithelium. The minor side effects of MAB 17-1A observed in clinical trials (1) strengthen our assumption of limited interaction with normal tissue.

Based on the 104-fold higher ADCC activity in vitro of c323/A3 compared to m17-1A and the equal safety of c17-1A compared to m17-1A in vivo, despite a 100-fold higher ADCC, it may be expected that the balance of efficacy versus safety of c323/A3 in vivo will be favorable, if ADCC is the mechanism of action of the MAB in vivo as well. Therefore, a reduced dose of c323/A3 may achieve effects similar to the 1-g doses of m17-1A currently used. Reduced doses of a chimeric MAB are also more cost effective and are expected to be less immunogenic and, therefore, could support prolonged treatment. Additionally, the excellent reactivity of MAB 323/A3 with a wide variety of different carcinomas indicates that MAB 323/A3 could be more widely applicable than MAB 17-1A, which has a low reactivity with a number of carcinomas (breast, lung, etc., see Fig. 1). The results presented in this study suggest that it is worthwhile to study whether chimeric MAB 323/A3 may be capable of significantly improving clinical results obtained to date with MAB 17-1A.

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