IN VITRO AND IN VIVO ACTIVITY OF MT201, A FULLY HUMAN MONOCLONAL ANTIBODY FOR PANCARCINOMA TREATMENT

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IN our study, a novel, fully human, recombinant monoclonal antibody of the IgG1 subtype is now well established for the treatment of human cancers. Such antibodies are effective as monotherapy and in combination with chemotherapy. Treatment of advanced breast cancer by the HER-2-specific trastuzumab (Herceptin®) and of follicular non-Hodgkin B-cell lymphoma by the CD20-specific rituximab (Mabthera®, Rituxan) was shown to lead to increased overall survival.1-2 A number of other promising IgG1 antibodies targeting EGFR for carcinoma and CD52, CD33 and CD22 for lymphoma and leukemia treatment are in late stages of clinical development.3 Human IgG1 is thought to eliminate tumor cells by CDC, ADCC and, depending on the target, additively by direct pro-apoptotic signaling or growth factor receptor antagonism. A recent study by Clynes et al.3 suggested that ADCD is a major in vivo mechanism of IgG1 action.

A murine monoclonal IgG2a antibody called edrecolomab (17-1A; Panorex®) was among the first monoclonal antibodies administered to humans for treatment of cancer.4-5 Murine IgG2a is the functional equivalent of human IgG1, which to some extent can compensate for the clinical deficiency of edrecolomab (Panorex®). MT201 exhibited Ep-CAM-specific CDC with a potency similar to that of edrecolomab. However, the efficacy of ADCC of MT201, as mediated by human immune effector cells, was by 2 orders of magnitude higher than that of edrecolomab. Addition of human serum reduced the ADCD of MT201 while it essentially abolished ADCC of edrecolomab within the concentration range tested. In a nude mouse xenograft model, growth of tumors derived from the human colon carcinoma line HT-29 was significantly and comparably suppressed by MT201 and edrecolomab. The fully human nature and the improved ADCD of MT201 with human effector cells will make MT201 a promising candidate for the clinical development of a novel pan-carcinoma antibody that is superior to edrecolomab.

The use of humanized or murine/human chimeric monoclonal antibodies of the IgG1 subtype is now well established for the treatment of human cancers. Such antibodies are effective as monotherapy and in combination with chemotherapy. Treatment of advanced breast cancer by the HER-2-specific trastuzumab (Herceptin®) and of follicular non-Hodgkin B-cell lymphoma by the CD20-specific rituximab (Mabthera®, Rituxan®) was shown to lead to increased overall survival.1-2 A number of other promising IgG1 antibodies targeting EGFR for carcinoma and CD52, CD33 and CD22 for lymphoma and leukemia treatment are in late stages of clinical development.3 Human IgG1 is thought to eliminate tumor cells by CDC, ADCC and, depending on the target, additively by direct pro-apoptotic signaling or growth factor receptor antagonism. A recent study by Clynes et al.3 suggested that ADCD is a major in vivo mechanism of IgG1 action.

A murine monoclonal IgG2a antibody called edrecolomab (17-1A; Panorex®) was among the first monoclonal antibodies administered to humans for treatment of cancer.4-5 Murine IgG2a is the functional equivalent of human IgG1, which to some extent can also exhibit ADCD and CDC with human effector cells and human complement, respectively. Edrecolomab was obtained by immunization of mice with human colon cancer cells and recognizes the pan-epithelial differentiation antigen Ep-CAM,7-10 which is widely involved in homotypic cell adhesion of epithelial cells. Since then, numerous clinical trials have been undertaken with edrecolomab and other Ep-CAM-specific murine, chimeric and humanized monoclonal antibodies of various affinities. Ep-CAM-specific antibodies were also tested preclinically and clinically in the form of conjugates with toxins, radioisotopes and the cytokines IL-2 and GM-CSF. This vast experience with monoclonal antibodies and derivatives against the Ep-CAM target has however not yet been translated into an established antibody-based therapy in the clinic. The only exception is edrecolomab, which obtained market approval for a limited number of years in Germany. In an academically sponsored phase III trial, monotherapy with edrecolomab was shown to increase the overall survival after 10 years by 32% in patients with resected colorectal carcinoma at the Dukes C stage.11-12 A recent, much larger phase III trial has shown that edrecolomab monotherapy was inferior after a 3-year observation period to the meanwhile introduced chemotherapy with 5-fluorouracil/leucovorin.13 This led to the withdrawal of edrecolomab’s market approval. Nevertheless, the edrecolomab monotherapy arm in the large phase III trial confirmed the overall survival observed in the Riehmüler study.14

A persistent issue with the pan-carcinoma target Ep-CAM is its presence on normal epithelia as a tissue-specific cell adhesion molecule.10-14 However, a recent study with transgenic mice expressing human Ep-CAM suggested that Ep-CAM is a valid tumor target based on its differential accessibility to parenterally administered Ep-CAM antibodies.15 I.v.-injected anti-human Ep-CAM antibody did homogenously stain human Ep-CAM-expressing syngenic tumors in transgenic mice while no significant binding of the antibody to the Ep-CAM present on normal tissue such as colon, pancreas or lung was detected. These normal tissues were however strongly stained with the human Ep-CAM-specific antibody on sections prepared for immunohistochemical analysis. Recent immunohistochemistry studies of tumor samples from prostate, gastric and head and neck cancers have shown that Ep-CAM expression can increase with disease progression.16-18 This apparent overexpression may further widen the therapeutic window of the Ep-CAM target in certain indications.

The low-affinity anti-Ep-CAM antibody edrecolomab is fairly well tolerated in humans where doses of several grams were given. Nevertheless, the edrecolomab monotherapy arm in the large phase III trial confirmed the overall survival observed in the Riehmüler study.14

Abbreviations: ADCD, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CHO, Chinese hamster ovary; ECACC, European Collection of Cell Cultures; ED50, half maximal dose; EGFR, epithelial growth factor receptor; Ep-CAM, epithelial cell adhesion molecule; E:T ratio, effector:target cell ratio; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte/macrophage colony stimulating factor; HER-2, human epithelial growth factor receptor-2; IL-2, interleukin-2; mAb, monoclonal antibody; MFI, mean fluorescence intensity; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PI, propidium iodide; PVP, polyvinylpyrrolidone; s.c., subcutaneous.

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occasionally given without causing serious side effects. By contrast, the high-affinity antibody 3622/W94 had a low maximum tolerated dose of 30 mg and caused pancreatitis at higher concentrations.\textsuperscript{20–22} Conjugates of other Ep-CAM antibodies with toxins also showed significant side effects as is expected for antibodies attacking epithelia.\textsuperscript{23} Edrecolomab and 3622/W94 were reported to bind the same subdomain of Ep-CAM,\textsuperscript{24} suggesting that affinity and not epitope recognition was responsible for the difference in tolerability. A drawback of edrecolomab for human therapy is its murine nature, resulting in a neutralizing immune response, short serum half-life, no option for chronic treatment and reduced compatibility with human immune effector mechanisms.

We therefore developed a fully human monoclonal antibody against Ep-CAM with the goal to preserve the safety profile of the low-affinity edrecolomab but to improve its antitumor activity. In our study, we characterized this novel antibody, called MT201 (HD69)\textsuperscript{25} and compared its \textit{in vitro} and \textit{in vivo} properties with those of edrecolomab. We anticipate that the human monoclonal antibody MT201 has the potential to significantly improve upon the clinically validated murine monoclonal antibody edrecolomab not solely with respect to better pharmacokinetic properties resulting from its fully human nature, but also with respect to its higher efficacy of tumor cell elimination via human effector cells.

**MATERIAL AND METHODS**

**Cell lines and PBMC**

CHO cells were purchased from the American Type Cell Culture Collection (ATCC, Rockville, MD). HT-29 cells, SW480, 22RV1 and LnCAP lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zelllinien (DSMZ, Braunschweig, Germany) and KATO III from the European Collection of Cell Cultures (ECACC, Salisbury UK). Cells were cultured as recommended by the suppliers. A CHO cell clone transfected with human Ep-CAM cDNA was produced as described.\textsuperscript{25} Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll density centrifugation from enriched lymphocyte preparations (buffy coats) obtained from local blood banks. PBMC were prepared on the same day of buffy coat receipt. Erythrocytes were removed from PBMC by erythrocyte lysis buffer (155 mM NH\textsubscript{4}Cl, 10 mM KH\textsubscript{C}\textsubscript{3} and 100 \textmu M ethylenediamine tetraacetic acid; EDTA) and thrombocytes removed via the supernatant obtained after centrifugation of PBMC at 100g for 10 min PBMC were typically used between days 2 and 4 after preparation without additional stimulation.

**Antibodies**

MT201 was produced at Micromet AG (Martinstied, Germany) by a CHO cell clone and purified to homogeneity by protein-A affinity and anion exchange chromatography.\textsuperscript{25} The murine monoclonal antibodies M79, 323/A3, 7C1 and 425 were kind gifts of Drs. P. Kufer and J. Johnson (Institute of Immunology, Munich, Germany). Edrecolomab (Panorex)\textsuperscript{25} was purchased from GlaxoSmithKline, Munich, Germany, Chimeric 17-1A called C46 was a kind gift of Dr. Hakan Mellstedt of Karolinska University, Stockholm, Sweden. FITC-labeled goat-anti-mouse IgG was from Pharmingen, Heidelberg, Germany (no. 34161D) and FITC-labeled goat-anti-mouse IgG (Pharmingen; each at a concentration of 25 mg/ml). Binding reactions with 10 different concentrations of MT201 were performed in 50 ml with 200,000 cells for 30 min at 4°C followed by 2 washes in FACS buffer. Secondary and tertiary antibodies were subsequently incubated for 30 min at 4°C followed by washes and flow cytometric analysis. The sandwich detection of MT201 was necessary to quantitate the number of bound MT201 by using the QIFIKIT kit (DAKO, Glostrup, Denmark; no. K0078). This kit allows one to quantitate by FACS the number of bound murine antibody to calibrated beads with 3,200–689,000 bound IgG molecules. A strictly linear relationship of MFI and bound IgG was obtained.

Data were analysed by Scatchard plotting and direct KD determination from binding curves. One hundred microliters of trypsinized, medium-washed KATO III cells was performed in a running buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant (P-20). KD and rate constants were determined from sensorgrams collected with 12 different antibody concentrations.

**CDC assay**

The FACS-based assay used 50,000 KATO III cells per reaction. One hundred microliters of trypsinized, medium-washed KATO III cells in RPMI/10% FCS were reacted with 20 mg antibody solution. The CDC reaction was started by addition of 80 mg of a solution containing 25% human serum in RPMI/10% FCS, resulting in a final concentration of 10% human serum with active complement. Human serum was collected from healthy donors. An aliquot was treated at 56°C and served as a control containing inactivated complement. Incubation was for 2 hr at 37°C in an atmosphere of 5% CO\textsubscript{2}. Cells were collected by centrifugation and resuspended in FACS buffer containing 1 mg/ml propidium iodide (PI). Ten thousand events were collected by flow cytometry. CDC was determined from the number of surviving PI-negative cells in gate R1.

**Binding studies**

Antibody binding to cells was studied by flow cytometry using a FACS CALibur instrument (Beckton Dickinson, Heidelberg, Germany) equipped with a 488 nm argon laser. Data were analyzed by CellQuest Software (Becton Dickinson). Cells were washed by FACS buffer containing phosphate-buffered saline, 1% fetal calf serum (FCS) and 0.05% sodium azide. Flow and cleaning solutions were purchased from Becton Dickinson and used according to the manufacturer’s instructions. FACS data were quantitated as histograms by determining the mean fluorescence intensity (MFI) as proposed by Diamond and Demaggio.\textsuperscript{26} A CHO cell clone transfected with human Ep-CAM cDNA-transfected CHO cells was performed in 30–50 \textmu M FACS buffer at room temperature for 30–60 min. Ten thousand events were recorded by FACS and MFI determined.

**Determination of binding constants**

For Scatchard analysis, the binding of MT201 to human gastric carcinoma line KATO III was titrated using FACS analysis essentially as described by Krause et al.\textsuperscript{27} MT201 was detected by a sandwich of unlabeled mouse-anti-human IgG (Pharmingen; no. 34161D) and FITC-labeled goat-anti-mouse IgG (Pharmingen; each at a concentration of 25 mg/ml). Binding reactions with 10 different concentrations of MT201 were performed in 50 ml with 200,000 cells for 30 min at 4°C followed by 2 washes in FACS buffer. Secondary and tertiary antibodies were subsequently incubated for 30 min at 4°C followed by washes and flow cytometric analysis. The sandwich detection of MT201 was necessary to quantitate the number of bound MT201 by using the QIFIKIT kit (DAKO, Glostrup, Denmark; no. K0078). This kit allows one to quantitate by FACS the number of bound murine antibody to calibrated beads with 3,200–689,000 bound IgG molecules. A strictly linear relationship of MFI and bound IgG was obtained.

Data were analysed by Scatchard plotting and direct KD determination from binding curves using the PRISM software program version 3.02 (GraphPad Software, San Diego, CA).

Biacore analysis was performed by Inven tus BioTec GmbH (Muenster, Germany) using a Biacore 2000 reader (Applied Biosystems, Uppsala, Sweden). Soluble, recombinant extracellular domain of human Ep-CAM was produced and purified from the supernatant of stably transfected CHO cells.\textsuperscript{25} Ep-CAM protein was coated to CMS flow cells (Becton Dickinson) using the Amine Coupling Kit as described by the manufacturer. Binding studies of MT201 and edrecolomab were performed in a running buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant (P-20). KD and rate constants were determined from sensorgrams collected with 12 different antibody concentrations.

**Binding studies**

Antibody binding to cells was studied by flow cytometry using a FACS CALibur instrument (Beckton Dickinson, Heidelberg, Germany) equipped with a 488 nm argon laser. Data were analyzed by CellQuest Software (Becton Dickinson). Cells were washed by FACS buffer containing phosphate-buffered saline, 1% fetal calf serum (FCS) and 0.05% sodium azide. Flow and cleaning solutions were purchased from Becton Dickinson and used according to the manufacturer’s instructions. FACS data were quantitated as histograms by determining the mean fluorescence intensity (MFI) as proposed by Diamond and Demaggio.\textsuperscript{26} Binding competition studies were done in 2 series. In 1 series, FITC-labeled antibodies (10 mg/ml) and varying concentrations of unlabeled competitor antibodies were premixed before addition to cells. In a second series, fluorescently labeled antibodies were allowed to first bind to cells at 4°C for 30 min before addition of unlabeled antibodies. Antibody binding to 120,000–200,000 human Ep-CAM cDNA-transfected CHO cells was performed in 30–50 \textmu M FACS buffer at room temperature for 30–60 min. Ten thousand events were recorded by FACS and MFI determined.
Immune effector cells (PBMC) were prepared as described above. One million target cells were labeled with 0.2 μM calcein AM (Molecular Probes, Göttingen, Germany; no. C-1430) for 30 min at 37°C in cell culture medium. After 2 washes in PBS, a cell density of 5 × 10^5 cells/ml was adjusted in RPMI/10% FCS and 100 μl aliquots of 50,000 cells used per assay reaction. Cultured PBMC were washed in PBS followed by RPMI/10% FCS and adjusted by dilution with RPMI/10% FCS to the desired E:T ratio. Antibodies were diluted in RPMI/10% FCS to the required concentration. If not otherwise indicated, a standard reaction at 37°C/5% CO₂ was for 4 hr and used 50,000 calcein-AM-labeled target cells, 1 million PBMC (E:T ratio of 1:20) and 20 μl antibody in a total volume of 100 μl. After the reaction, cells were collected by centrifugation and resuspended in FACS buffer containing 1 μg/ml PI. One hundred thousand events were collected by flow cytometry.

Quantitation of cytotoxicity was based on the number of alive and dead target cells in the control reaction without antibody. This was necessary because effector cells tend to take up calcein AM released from dead target cells, in particular, during longer incubation periods. Where indicated, either specific or overall ADCC was determined. Sigmoidal dose response curves typically had r² values >0.95 as determined by Prism Software.

Animal experiments

In-house bred, male athymic NMRI:nu/nu mice with a body weight of 30 ± 1 g and an age of 6–8 weeks were used for testing the effect of MT201, edrecolomab and C46 on tumor growth. The mice were held under sterile standardized conditions. These were 22°C, 50 ± 5% relative humidity, a 12 hr light/dark rhythm, autoclaved food and bedding (by SSniff, Soest, Germany) and ad libitum acidified tap water. All animal experiments were performed according to the German Animal Protection Law with permission from the responsible local authority. HT-29 colon carcinoma cells were produced by cell culture. One million cells in a volume of 0.1 ml PBS were injected into the left flank of nude mice on day 0. Treatment with antibodies at 30 μg in 0.1 ml PBS started on day 1 after tumor-cell inoculation and was repeated at days 4 and 7. Antibodies were administered by the tail vein. As controls, PBS and rituximab (30 μg/dose) were used. Statistical analysis of tumor growth was performed by the Mann-Whitney test. The significance level was p ≤ 0.05.

RESULTS

Binding specificity of MT201

The cell-binding properties of MT201 were investigated by flow cytometry. A cell line not expressing human Ep-CAM (chinese hamster ovary, CHO), CHO cells stably transfected with human Ep-CAM cDNA (Ep-CAM-CHO) and a human gastric carcinoma line naturally expressing Ep-CAM (KATO III) were tested for MT201 binding. Figure 1a shows FACS histograms of the 3 cell lines in the presence of 1.6 and 100 μg/ml MT201, as detected by a secondary FITC-labeled mouse-anti-human IgG. Controls (left histograms) used the secondary antibody in the absence of MT201. Cell counts are plotted against the fluorescence intensity of cells in the FITC channel. Light gray, controls; middle gray, 6.3 μg/ml MT201; black, 100 μg/ml MT201. (b) Quantitation and dose-response analysis. Mean fluorescence intensity (MFI) of histograms from triplicate determinations is shown plotted against the indicated concentrations of MT201.

was presumably mediated by Fcγ receptor binding of MT201 to those immune cells (data not shown).

Low-affinity Ep-CAM binding of MT201 is similar to that of edrecolomab

Binding constants for MT201 were determined by both a FACs-based binding assay using KATO III cells and a plasmon resonance-based assay with the Biacore analyzer. Scatchard analysis of cell binding data yielded a mean dissociation constant for MT201 of 1.38 × 10⁻⁷ M (r² = 0.9423). Biacore analysis using sensor chips coated with the recombinant extracellular domain of human Ep-CAM yielded a dissociation constant of 1.75 × 10⁻⁷ M, a k_on of 2.63 × 10⁵ M⁻¹s⁻¹ and a k_off of 4.61 × 10⁻² s⁻¹. With these affinity and rate constants, MT201 exhibited similar binding characteristics as the clinically validated murine monoclonal antibody edrecolomab. Under identical Biacore assay conditions, a dissociation constant of 1.75 × 10⁻⁷ M, a k_on of 2.63 × 10⁵ M⁻¹s⁻¹ and a k_off of 4.61 × 10⁻² s⁻¹. With these affinity and rate constants, MT201 exhibited similar binding characteristics as the clinically validated murine monoclonal antibody edrecolomab.
Epitope recognition of MT201 relative to other anti-Ep-CAM mAbs

To investigate the relatedness of epitopes, the binding of MT201 to Ep-CAM-transfected CHO cells was studied in the presence of increasing amounts of 3 other Ep-CAM-specific monoclonal antibodies. The 3 other mAbs were M79, edrecolomab and 323/A3, a high-affinity murine monoclonal antibody used to develop the clinically tested, humanized monoclonal antibody W94.

Each antibody was labeled by activated fluorescein and binding competition studied by FACS in the presence of increasing concentrations of respective unlabeled antibodies either after prebinding of the FITC-labeled antibody to cells at 4°C (Fig. 2, right panels) or by simultaneous addition of FITC-labeled and unlabeled antibodies (left panels). Murine antibodies against HER-2 (7C1) or EGFR (mAb 425) served as nonspecific controls and did not show significant binding competition throughout the experiment (Fig. 2).

Binding of FITC-labeled MT201 was best competed by 323/A3 followed by M79 and self (Fig. 2a,b). Edrecolomab exhibited the weakest inhibition of MT201 binding. Cell binding of FITC-labeled M79 was very similarly competed by MT201, edrecolomab and self and best by 323/A3 (Fig. 2c,d). FITC-labeled edrecolomab was well competed by all antibodies except for MT201 (Fig. 2e,f). Binding of FITC-labeled 323/A3 was very weakly competed by the low-affinity antibodies MT201, edrecolomab and M79, but was efficiently competed by self (Fig. 2g,h).

In conclusion, the mutual competition of all 4 tested antibodies suggests their binding to a common subdomain of the extracellular domain of human Ep-CAM. The efficiency and dose-dependence of binding competition was consistent with the relative affinities of mAbs for Ep-CAM. For 323/A3 and edrecolomab, the subdomain of Ep-CAM was previously determined to be the immunodominant, outer EGF-like domain I. Because both M79 and 323/A3 did compete efficiently with all other antibodies, it appears they recognized epitopes on the same Ep-CAM subdomain that are overlapping with those recognized by MT201 and edrecolomab. Within this Ep-CAM subdomain, MT201 and edrecolomab apparently recognized the most distant epitopes, as was evident from their weakest cross-competition among the 4 anti-Ep-CAM mAbs tested.

MT201 and edrecolomab exhibit comparable CDC

CDC of MT201 was investigated in a FACS-based assay (Fig. 3). This assay monitors the lysis of KATO III cells by the uptake of propidium iodide (PI). At a final human serum concentration of 10%, a significant and dose-dependent tumor cell lysis was observed in the presence of 6.3 or 100 μg/ml MT201 (Fig. 3a). This was evident by the appearance of a PI-positive cell population and the concomitant disappearance of the PI-negative alive cell population in the shown gate. Heat-inactivated human serum did not lead to significant cell lysis in the presence of 100 μg/ml MT201.
The human IgG1 MT201 and the murine IgG2a edrecolomab exhibited a comparable dose response of CDC with human serum. Both antibodies reached half-maximal cell lysis at approximately $10^{-8}$ M MT201 in the upper half of the plot. (a) Comparison of MT201 CDC with that of edrecolomab and an isotype control (rituximab). KATO III cells were incubated with the indicated concentrations of antibodies in the presence of human serum and target cell elimination quantitated from FACS data (see a). (b) CDC of MT201 with CHO, human Ep-CAM cDNA-transfected CHO and KATO III cells. Error bars give standard deviations from triplicate determinations.

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ADCC of MT201

We established a FACS-based ADCC assay monitoring the fate of target cells over the high background of human PBMC used as effector cells. To this end, KATO III cells were loaded with the fluorescent dye calcein AM, which is trapped inside live cells through esterase-mediated cleavage. Labeled, live target cells could be efficiently separated in FL-1 from unlabeled PBMC (Fig. 4a, right gate). PI allowed for separation and quantitation of calcine AM-labeled dead target cells in the upper gate (Fig. 4a). Upon incubation with MT201 at an effector:target cell (E:T) ratio of 20:1, a dose-dependent decrease of live cells in the right gate and a concomitant increase of PI-positive cells in the upper gate was observed. A short 4 hr assay period was chosen to also observe distinct levels of overall tumor cell lysis in the plateau phase of dose-response plots.

ADCC by MT201 was specific. Figure 4b shows that no ADCC was observed with CHO cells unless this rodent cell line was stably transfected with human Ep-CAM cDNA. In contrast to CDC (Fig. 3), KATO III cells were significantly more sensitive to ADCC than Ep-CAM-transfected CHO cells (Fig. 4b). The half-maximal doses (ED$_{50}$ values) for ADCC were below $10^{-8}$ M MT201 in the absence of effector cells. With increasing ratios of human PBMC, the extent of cell lysis, as seen in the plateau phase, increased. Although it doubled with an E:T ratio going from 10:1 to 20:1, not much of an increase was seen upon a further doubling to 40:1. The ED$_{50}$ values of ADCC were not strongly affected by the various E:T ratios.

PBMC donor variation strongly affects ADCC

PBMC were prepared from various healthy donors and all tested for ADCC in the presence of MT201 at the same E:T ratio of 20:1. Donor PBMCs exhibited a significant variation of ADCC with respect to the extent of nonspecific and specific tumor cell lysis, as well as ED$_{50}$ values. Figure 5a compares the dose-response behaviour of ADCC by 6 distinct donor PBMCs. More than 10-fold variations among donors were observed. A statistical analysis (Fig.
5b) compares ED<sub>50</sub> values obtained from dose-response analyses of a total of 23 healthy donors. Two-thirds of donor PBMCs gave ED<sub>50</sub> values between 100 and 500 ng/ml MT201 and only 4 donor PBMC gave ED<sub>50</sub> values /H11022<sub>1</sub>/g/ml. MT201 exhibits much higher ADCC than edrecolomab ADCC of MT201 and edrecolomab was compared using KATO III as target cells (E:T ratio of 20:1 in a 20 hr assay). A highly active donor PBMC was selected to see an optimal performance of edrecolomab. These PBMC gave an ED<sub>50</sub> value of ADCC for MT201 below 1 ng/ml (Fig. 5a). Under identical conditions, edrecolomab exhibited an ED<sub>50</sub> value /H11022<sub>100</sub>/g/ml. A similarly large difference in activity between MT201 and edrecolomab was seen with less-active PBMC effectors (data not shown).

Upon addition of 50% human serum, a source of extra human IgG, the ADCC of MT201 was reduced by 2 orders of magnitude from an ED<sub>50</sub> value of 1 ng/ml to approximately 100 ng/ml (Fig. 5b). Under identical conditions, human serum essentially abolished the ADCC of edrecolomab (Fig. 5c). Significant cell lysis was seen up to 100 ng/ml edrecolomab, a concentration of the antibody that is unlikely to be exceeded much in vivo.

**Inhibition of tumor growth by MT201 in a nude mouse model**

The *in vivo* efficacy of MT201 against tumors was investigated in a nude mouse model using the Ep-CAM-positive human colon carcinoma line HT-29. KATO III cells, which worked well *in vitro* as a target cell line, did not develop tumors in nude mice. MT201 or controls were administered via the tail vein on days 1, 4 and 7 following the subcutaneous (s.c.) injection of /1 × 10<sup>6</sup>/ HT-29 tumor cells. Solid-tumor growth to a mean size exceeding 0.35 cm<sup>3</sup> was observed with both the PBS control and an IgG1 isotype control not recognizing HT-29 cells (*i.e.*, CD20-specific rituximab) (Fig. 7a). Administration of 3 times 30 μg MT201 led to a statistically significant inhibition of tumor growth (*p* = 0.05) that lasted for the entire observation period of 37 days. Three times 3 μg MT201 also caused a reduction in tumor growth but the effect did not reach statistical significance. The 30 μg dose of MT201 was diluted for treatment of mice either in PBS or in a buffer containing 1% of the excipient polyvinylpyrrolidone (PVP-17) that was used for formulation of clinical test material. PVP did not significantly affect the *in vivo* efficacy of MT201.

The *in vivo* efficacy of MT201 was compared to that of murine edrecolomab (IgG2a) and C46, a chimerized version of edrecolomab with a human IgG1 backbone. Thirty-microgram doses of all 3 antibodies given on days 1, 4 and 7 after s.c. inoculation of 10<sup>6</sup> tumor cells did inhibit tumor growth in the HT-29 nude mouse model (Fig. 7b). No statistically significant difference was obtained when the activities of 3 antibodies were compared among each other. There was, however, a statistically significant difference when the activity of the antibodies was compared to the PBS and IgG1 isotype (rituximab) controls. Edrecolomab and MT201 gave a number of significant readings (*p* ≤ 0.05; marked by asterisks), whereas C46 exhibited only a significant reading on day 21. The difference between edrecolomab and C46 may reflect the different efficiency of murine IgG2a and human IgG1 Fc effector domains in a murine background. MT201, which performed in the mouse background similarly well as edrecolomab, may thus be in

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**FIGURE 4** – Antibody-dependent cellular cytotoxicity (ADCC) of MT201. (a) FACS-based assay. KATO III target cells were fluorescently labeled by calcine AM to distinguish them from the background of PBMC used as effector cells. Alive KATO III cells in gate R1 were distinguished by flow cytometry from MT201-eliminated cells by additional labeling with propidium iodide (PI) in gate R2. The effect of the indicated concentrations of MT201 in a 4 hr assay on the viability of KATO III cells at an E:T ratio of 20:1 is shown. (b) Dose-response analysis. KATO III, CHO and human Ep-CAM cDNA-transfected CHO cells were tested at the indicated concentrations of MT201 for their susceptibility to ADCC at an E:T ratio of 20:1. (c) The effect of varying E:T ratios on the efficacy of ADCC by MT201. A 4 hr ADCC assay was performed at the indicated concentrations of MT201 and E:T ratios. Error bars indicate standard deviations from triplicate determinations.
DISCUSSION

In our study, we characterized in detail the human monoclonal antibody MT201 for Ep-CAM specificity binding, epitope recognition in relationship to other well-characterized anti-Ep-CAM monoclonal antibodies, CDC and ADCC and inhibition of tumor take in a nude mouse model. The in vitro and in vivo data suggest that the human monoclonal antibody MT201 has characteristics desirable for the development of a next-generation anti-Ep-CAM antibody: low affinity, potent ADCC with human effector cells, CDC activity and inhibition of tumor growth in vivo. Moreover, the overall sequence identity of 95% of VH and VL domains of MT201 to human germline sequences would predict a very low immunogenicity in man.

Despite many disappointing efforts, Ep-CAM still appears as an attractive pan-carcinoma target for antibody-based therapies. Unlike the clinically validated antibody targets HER-2 and EGFR, Ep-CAM is not overexpressed on tumor cells as a consequence of gene amplification but, as an epithelial differentiation antigen, is constitutively expressed on almost all carcinomas. HER-2-positive breast tumors only occur in 15–30% of patients. The frequency of EGFR overexpression in breast cancer is ca 40%, and somewhat higher in other tumors. By contrast, Ep-CAM expression on certain tumors such as prostate and gastric carcinoma reaches 100% as examined by immunohistochemistry. Moreover, Ep-CAM was found to be significantly upregulated with disease progression in several cancers including breast cancer, suggesting that Ep-CAM expression on tumor cells confers a growth, invasion and/or survival benefit. A recent study showed that Ep-CAM can interact with the negative regulatory receptor LAIR-1 present on most immune cells. This indicates that Ep-CAM expression may confer a general suppression of immune cell activity in the microenvironment of the tumor. The widespread expression of Ep-CAM with respect to carcinoma type, frequency in patient population and on cells of a given tumor make Ep-CAM a unique and promising pan-carcinoma target. Additional efforts are thus warranted to optimally exploit the therapeutic window offered by the differential accessibility of Ep-CAM to antibodies, its widespread expression and its upregulation on certain tumors.
Body edrecolomab and its unique properties are pointing the way and small tumor size do not overburden the limited effect. Minimal residual disease (micrometastasis) where low tumor load reduces tumor load and increase the tumor of cancer, a combination of IgG with chemotherapy may help penetration capacity of "naked" IgG. With more advanced stages of cancer, a combination of IgG with chemotherapy may help reduce tumor load and increase the tumor's accessibility for antibodies. Currently, MT201 is the only anti-Ep-CAM monoclonal antibody of human origin under clinical development. Its VH domains were isolated from a human B-cell repertoire that has not yet undergone somatic mutations and deletion of autoreactive specificities. We determined by Scatchard and Biacore analysis an affinity of MT201 for Ep-CAM in the 100 nM range. This affinity for Ep-CAM was only slightly higher than that of edrecolomab. We thus reached our goal of obtaining a fully human IgG1 with an affinity close to that of the clinically validated edrecolomab.

Comparison analysis of MT201 with the Ep-CAM-specific monoclonals edrecolomab, M79 and 323/A3 suggests that all 4 monoclonal antibodies recognize human Ep-CAM in a very related fashion. We observed that edrecolomab binding was efficiently competed by 323/A3 and vice versa, suggesting that their epitopes on the EGF-like domain I are essentially overlapping. The distinct toxicity of the 2 antibodies in man may thus be related to their >100-fold difference in affinity rather than the recognition of distinct epitopes. High-affinity anti-Ep-CAM antibodies may interfere as antagonistic autoantibodies with the adhesion function of Ep-CAM in normal epithelium, resulting in perturbed epithelial integrity. The increased levels of pancreatic enzymes in blood observed with the humanized version of 323/A2 would be consistent with this hypothesis. Epithelial damage by the humanized version of 323/A3 could also be a consequence of CDC- or ADCC-mediated damage to epithelial cells. However, this should then also be observed for edrecolomab, which does not significantly increase amylase levels in blood.

Although edrecolomab and MT201 only very weakly competed each other for their binding to Ep-CAM, both were well competed by 323/A3 and M79. This indicated that the epitopes recognized by MT201 and edrecolomab were closely adjacent though not strictly overlapping. Binding studies with truncated Ep-CAM showed that both 323/A3 and edrecolomab do bind the immunodominant EGF-like domain I of Ep-CAM. We infer from the competition data that M79 and MT201 also recognized this subdomain, although this conclusion may require further support from binding studies using truncated Ep-CAM versions.

Although the cytotoxicity of MT201 mediated by ADCC and CDC was substantial, MT201 alone did not show any detectable effect on the viability of Ep-CAM-positive human tumor cell lines in the absence of complement (human serum) or human immune effector cells. The monoclonal antibody does therefore not belong to the class of antitumor antibodies that directly elicit pro-apoptotic signaling or growth inhibition by recognition of a growth factor receptor. We do not know whether MT201 binding to Ep-CAM produces any kind of intracellular signal and it is currently not known whether any of the other anti-Ep-CAM antibodies does. It is interesting to note that in vitro studies suggested that both trastuzumab and rituximab render tumor cells apoptotic by inhibition of growth and survival signals. However, in Fcγ KO mice rendered incapable of ADCC, both antibodies lost most of their antitumor activity against tumor cell lines that are otherwise susceptible to the pro-apoptotic activity of the antibodies in vitro.

We noted a substantially higher ADCC of MT201 compared to edrecolomab. This may not be related to the slightly higher binding affinity of MT201 because the CDC activity of the 2 antibodies was very similar. We rather attribute the substantial difference in ADCC to the class of antitumor antibodies that directly elicit pro-apoptotic activity of the antibodies.

The vast clinical experience with the murine monoclonal antibody edrecolomab and its unique properties are pointing the way to an optimized Ep-CAM-specific antibody therapy. Ep-CAM-specific human IgG1 appears particularly useful for treatment of minimal residual disease (micrometastasis) where low tumor load and small tumor size do not overburden the limited efficacy and penetration capacity of "naked" IgG. With more advanced stages of cancer, a combination of IgG with chemotherapy may help reduce tumor load and increase the tumor's accessibility for antibodies. Currently, MT201 is the only anti-Ep-CAM monoclonal antibody of human origin under clinical development. Its VH domain was isolated from a human B-cell repertoire that has not yet undergone somatic mutations and deletion of autoreactive specificities. We determined by Scatchard and Biacore analysis an affinity of MT201 for Ep-CAM in the 100 nM range. This affinity for Ep-CAM was only slightly higher than that of edrecolomab. We thus reached our goal of obtaining a fully human IgG1 with an affinity close to that of the clinically validated edrecolomab.
cross species barriers. Pharmacokinetic behaviour adds even more differences to the 2 systems. Compared to human IgG1, the serum half-life of murine IgG2a within humans is markedly reduced apparently because of its low affinity to FcRn.41 Likewise, the half-life of human IgG isotopes is much reduced in mice.42 On these notions, a direct comparison between edrecolomab and MT201 in a mouse background seems to provide limited information with respect to the situation in humans. The animal data would predict that MT201, which in mice was similarly potent as edrecolomab, may perform far better in humans because of a longer half-life and improved ADCC with human effector cells. Our in vitro ADCC experiment in the presence of human serum and testing human effector cells from various donors may have a better predictive potential about the in vivo efficacy of MT201 (and edrecolomab) in humans than the nude mouse model.

A chimeric version of edrecolomab with a human IgG1 Fc part called C463 was clinically tested in combination with the immuno-stimulatory cytokine GM-CSF.43,44 As expected, the incidence of an immune response against the chimeric form was increased compared to the murine version.45 In the presence of GM-CSF, of note, dosing and side effects associated with the chimeric version were not reported to be strikingly different from those of murine edrecolomab, although the chimeric edrecolomab is expected to better interact with human immune cells and therefore has improved ADCC activity. This supports the notion that side effects of high-affinity Ep-Cam monoclonal antibodies are associated with an antagonistic effect on Ep-Cam function rather than increased ADCC or CDC activity against epithelium. An ongoing phase I trial with MT201 will reveal the side effect profile of MT201, its immunogenicity, serum half-life and perhaps a maximally tolerated dose.

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