Tumor-Specific Inhibition of Membrane-Bound Complement Regulatory Protein Crry with Bispecific Monoclonal Antibodies Prevents Tumor Outgrowth in a Rat Colorectal Cancer Lung Metastases Model

Kyra A. Gelderman,1 Peter J. K. Kuppen,2 Noriko Okada,3 Gert Jan Fleuren,1 and Arko Gorter1

Departments of 1Pathology and 2Surgery, Leiden University Medical Center, Leiden, The Netherlands, and 3Department of Molecular Biology, Nagoya City University, Graduate School of Medical Science, Nagoya, Japan

ABSTRACT

Membrane-bound complement regulatory proteins (mCRP) inhibit complement-mediated tumor cell eradication in vitro and in vivo. Immunotherapy of cancer with monoclonal antibodies (mAbs) that activate complement might be hampered by expression of mCRP on tumor cells. An important strategy to improve mAb immunotherapy can be blocking or overwhelming mCRP at the tumor cells surface in a tumor-specific manner. In our study, we investigated the feasibility of this approach in vivo using bispecific mAbs (bi-mAbs). This study, performed in a syngeneic lung metastases model of rat (WAG/Rij) colorectal cancer, showed that modulation of mCRP on rat tumors resulted in significantly decreased tumor outgrowth. Opossonization of tumor cells with a bi-mAb directed against a tumor-associated antigen and rat mCRP Crry (MG42a*I2) almost completely prevented the outgrowth of lung tumors (0–7 tumors/rat; n = 17). Opossonization with mAb-cobra venom factor conjugates significantly reduced the number of lung tumors (23–59 tumors; n = 12) compared with the unconjugated MG42a (175–246 tumors; n = 17; P = 0.008 and 0.014, respectively). The effect of MG42a*I2 was shown to be increased by increased complement activation due to inhibition of Crry. Moreover, prophylactic treatment with MG42a*I2 or MG42a showed comparable results (3–24 and 215–472 tumors, P = 0.02; n = 6) as observed with pre-opsonized tumor cells without noticeable side effects, despite binding of MG42a*I2 to endothelium and leukocytes. These results demonstrate that Crry inhibits complement-mediated tumor cell eradication by immunotherapeutic mAbs and show that tumor-specific inhibition of complement regulatory proteins using bi-mAbs can significantly improve mAb-mediated immunotherapy.

INTRODUCTION

Tumors have developed several strategies to circumvent the host immune system. One of these strategies is to overexpress membrane-bound complement regulatory proteins (mCRP) that inhibit complement activation (1–3). Because complement activation can be an important effector mechanism of monoclonal antibody (mAb)-mediated immunotherapy of cancer, mCRP expression might hamper the therapeutic effect (4). This might be an explanation for the until now disappointing results of immunotherapeutic mAbs in the clinic (5). That these immunotherapeutic mAbs were previously successful in animal models can be explained by the use of tumor xenograft models and the observation that mCRP operate in a species selective fashion (6, 7). This underlines the need for appropriate animal models, with mCRP and complement of the same species, to investigate the effect of mCRP on cancer immunotherapy with mAbs.

In humans, there are three important mCRP expressed by tumor cells. CD46 is a cofactor for factor I-mediated cleavage of C3b and C4b, and CD55 accelerates the decay of C3 and C5 convertases (8). Both CD46 and CD55 inhibit the formation of the chemoattractants C3a and C5a and the deposition of C3b (further converted to iC3b) on the cell surface, thereby preventing attraction and activation of effector cells expressing Fc and complement receptors and subsequent complement-mediated cellular cytotoxicity (9). CD59 inhibits the formation of the membrane attack complex, thereby preventing direct complement-mediated lysis (8). In addition to the above-described mCRP, rodents express an additional C3 regulatory protein Crry/p65 (Crry), whereas CD46 expression is restricted to the testes. Crry is a functional homologue of CD46 and CR1 (10), thus acting at the level of C3, similar to human CD46 and CD55. Crry has been shown to be the most important C3 regulatory protein on tumor cells in rats (11, 12). Therefore, modulation of Crry on tumor cells is a suitable model for the modulation of human membrane-bound C3 regulatory proteins.

Previously, it has been shown that systemic inhibition of Crry by i.p. injection of anti-Crry mAbs in rats results in severe side effects, including endothelial damage, peritoneal hemorrhage, and death (13), as a result of unhampered massive complement activation, due to the ubiquitous expression of Crry. Therefore, when therapeutically inhibiting Crry on tumor cells, anti-Crry mAbs cannot be administered systemically, and manipulation should be primarily restricted to the tumor cells. To obtain this goal, we have investigated the feasibility of using bispecific mAbs (bi-mAbs) that both recognize a tumor antigen and Crry in a syngeneic rat model for colorectal cancer.

Previously, we have described that bi-mAb directed against Ep-CAM and CD55 as well as anti-Ep-CAM-CVF conjugates causes increased amounts of C3 deposition on human colorectal tumor cells in vitro as compared with the anti-Ep-CAM mAb alone (14). In the present study, to follow up on these in vitro studies, we have investigated the effect of blocking or overwhelming the effect of Crry on in vitro opsonized rat colon carcinoma cells in a syngeneic rat colorectal cancer lung metastases model. Blocking the function of Crry was achieved by using bi-mAbs, which possessed an arm recognizing a rat tumor antigen for tumor-specific homing and an arm that blocked Crry (15, 16). Overwhelming the function of Crry was achieved by tumor-specific targeting of cobra venom factor (CVF) with mAb-CVF conjugates (17). Control bi-mAb and anti-Crry F(ab)2 fragments were used to show that the observed effect of bi-mAbs was indeed due to the modulation of Crry. Finally, to mimic adjuvant mAb immunotherapy a prophylactic experiment was performed, to substantiate the in vivo observations in a therapeutic setting.

MATERIALS AND METHODS

Antibodies and Sera. The cell line producing 5I2 (IgG1), a blocking mAb directed against Crry, was a kind gift from Prof. Dr. H. Okada (Nagoya City University School of Medicine, Japan). The MG42a (IgG2a) and CC52 (IgG1) mAb directed against the rat colon adenocarcinoma cell line CC531 were produced as previously described (11, 18). FITC-conjugated goat anti-mouse IgG/γM was obtained from DAKO A/S (Glostrup, Denmark). FITC-conjugated goat antirat C3 was purchased from ICN Biomedicals, Inc. (Aurora, OH). Rat antirat IgG1 (Sanbio, Uden, The Netherlands) and goat antirat IgG2a

Received 7/16/03; revised 2/18/04; accepted 4/7/04.

Grant support: Dutch Cancer Society Grant 99032.

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Requests for reprints: Arko Gorter, Department of Pathology, L1-Q, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Phone: 31-71-526-6631; Fax: 31-71-5248158; E-mail: A.Gorter@lumc.nl.

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conjugated with horseradish peroxidase (Southern Biotechnology Associates) were used for the bi-isotopic ELISA.

Quadroma cells producing bi-mAb MG4₅⁺S12 and MG4⁺CC52 were obtained by fusion of the respective hybridomas (18, 19) and tested and purified as described before (16): The fractions that were eluted from protein A with pH 5.5 were tested for bi-isotopicity with a sandwich ELISA. Fractions were incubated on a plate coated with rat antimouse IgG1 and detected with goat antimonus IgG2a conjugated with horseradish peroxidase as described with SDS-PAGE on 7.5% gels under nonreducing conditions.

Bispecificity of bi-isotopic mAb was tested with a conjugation test: CC531 cells preincubated with S12 or CC52 (F(ab)₂) fragments were opsonized with bi-mAbs and respectively exposed to LT12 cells that expressed Crry but no MG4 or to CC531 cells opsonized with MG4 F(ab)₂ fragments. Whereas approximately 25% of cross-linking of these cells was observed with flow cytometry, bi-mAbs were considered to contain mAbs that recognized the two antigens (16).

Syngeneic normal rat serum was prepared by centrifugation on 4°C of coagulated blood of WAG/Rij rats. F(ab)₂, Fragments. F(ab)₂, fragments were produced as previously described previously (20). In short, 9 units of mAb were incubated with 1 unit of 1 M citrate and 2 units of 0.1 M HCl together with 100 units of pepsin-agarose as described previously (20). In short, 9 units of mAb were incubated with 1 unit of 1 M citrate and 2 units of 0.1 M HCl together with 100 units of pepsin-agarose supplied every other day for 2 weeks.

Tumor Cells. The CC531 cell line (21) was cultured in RPMI 1640 culture supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 50 μg/ml streptomycin, and 50 units/ml penicillin (all from Life Technologies, Paisley, United Kingdom). Tumor cells used for in vitro experiments were kept in culture for several passages. Cells used in vivo were all from the same passage and always cultured according to a standard schedule according to which cells were passaged every 4 days and fresh medium was supplied every other day for 2 weeks.

mAb-CVF Conjugates. CVF was isolated and tested for hemolytic activity as described before as was the conjugation of CVF to mAb (14, 17). In short, 5 mg of antibody and 2.5 mg of CVF were incubated with 0.1% (w/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 50 μg/ml streptomycin, and 50 units/ml penicillin (all from Life Technologies, Paisley, United Kingdom). Tumor cells used for in vitro experiments were kept in culture for several passages. Cells used in vivo were all from the same passage and always cultured according to a standard schedule according to which cells were passaged every 4 days and fresh medium was supplied every other day for 2 weeks.

Complement Activity ELISA. Conjugate (20 μg/ml) or mAb (10 μg/ml) was coated in 0.1 M NaHCO₃ buffer (pH 9.6) for 2 h at 37°C. The reaction was stopped with an equal volume of 3 M Tris. The reaction volume was adjusted for 5 min at 7,000 × g, and the F(ab)₂ containing supernatant was dialyzed against PBS. No fragments other than the F(ab)₂ fragments were detected in SDS-PAGE. The amount of released hemoglobin was measured at A₄₁₂.

Animal Experiments. Male WAG/Rij rats, a Wistar-derived inbred strain, were purchased from Charles River (Schoenefeld, Germany) and used at an average weight of 230 g. The animals had free access to standard food pellets and water. Animal experiment protocols were approved by the local university animal ethical committee. Tumor cells were harvested from a solution of 0.1% (w/v) EDTA and 0.25% (w/v) trypsin in Hanks’ buffered salt solution. After washing twice in PBS, 30 × 10⁶ cells were incubated with 4 ml of PBS containing either mAb (150 μg/100 pmol), F(ab)₂ (100 μg/100 pmol), bi-mAb (400 μg/269 pmol), or mAb conjugate (300 μg/100 pmol) for 1 h at 4°C. The ratio of mAbs and cells was comparable with the flow cytometry protocol. After incubation, cells were washed three times with PBS, adjusted to a concentration of 20 × 10⁶ cells/ml, and kept in suspension on ice until use while occasionally shaking. Rats were anesthetized with halothane, and 200 μl of tumor cell suspension, containing 4 × 10⁶ cells, were injected into the penile vein. In the prophylactic experiment, rats at t = −6 h received injections i.p. of 300 μg (200 pmol) of bi-mAb or 150 (100 pmol) of MG4 (amounts corrected for comparable binding sites to MG4) in 500 μl PBS. At t = 0 h, 4 × 10⁶ tumor cells were injected in the penile vein. At t = 6 h, a second comparable dose of mAb was administered. Blood was drawn from the tail vein at t = 0 and t = 18 to collect peripheral blood lymphocytes and serum. At day 21, rats were sacrificed by abdominal bleeding under halothane anesthesia. Lungs received injections of 15% Indian ink (Royal Talens, Apeldoorn, The Netherlands) in water via the trachea until completely filled. Lungs were removed, washed for 5 min in water, and fixed in Fekete’s solution containing 60% ethanol, 9% formaldehyde solution (4%), and 4.5% acetic acid (24). After 24 h of fixation, lungs were rinsed and stored in water. All macroscopically visible tumors on the lung surface were counted for each pair of lungs by an observer blinded for the treatment of the tumor cells or rats. The number of metastases was previously shown to correlate well with the total number of tumors present in the lungs (24, 25). Each experiment consisted of five rats per group and was repeated three or four times (total n = 12–17/group in total). For the therapeutic experiment, n = 6 rats/group. Differences between groups were statistically analyzed using a Mann-Whitney U test. Two of seven bi-mAb-injected rats were sacrificed 2 days after mAb injection to investigate biodistribution of the bi-mAb. Sections of liver, lung, colon, and skin were stained with goat antimonus-FITC, and staining levels were analyzed by confocal microscopy as described before (11).

Statistical Analysis. Data are given as group means ± SD. Differences in group means were tested for significance using Mann-Whitney U test, considering P < 0.05 significant.
RESULTS

Complement-Activating Capacities of MG42a, MG42a-CVF, and MG42a*5I2. With a complement activation ELISA, the complement-activating capacities of MG42a and MG42a-CVF were compared by detecting the amount of deposited C3. The amount of C3 deposition induced by MG42a-CVF was approximately 10 times higher compared with unconjugated MG42a (Fig. 1A). Using flow cytometry, opsonization with MG42a-CVF increased the amount of C3 deposition on CC531 cells by 100% compared with MG42a. The amount of C3 deposition induced by MG42a-CVF could still be increased another 300% compared with MG42a-CVF by simultaneously blocking Crry with 5I2 F(ab)_2 fragments, indicating the important role of Crry in controlling complement activation at the cell surface (Fig. 1B).

Next, a comparison was made between the amount of C3 deposition on CC531 cells induced by bi-mAb MG42a*5I2, by the parental MG42a mAb combined with 5I2 F(ab)_2, and by MG42a mAb alone (Fig. 1B). MG42a*5I2 and the two parental mAbs [MG42a and 5I2 F(ab)_2] were similar in their complement-activating capacities as measured by flow cytometry. F(ab)_2 of 5I2 were used, because the Fc part of 5I2 could, despite its IgG1 isotype, activate the rat complement system via the classical pathway (Ref. 26; data not shown). That the beneficial effect of bi-mAb or MG44 + 5I2 was dependent on the complement-activating MG44 antitumor arm was supported by the observation that 5I2 F(ab)_2 in combination with non-complement-activating MG44 IgG1 did not increase C3 deposition compared with MG42a alone. C3 deposition induced by bi-mAb or by both parental mAbs [MG44 IgG2a + 5I2 F(ab)_2], however, was significantly higher compared with MG42a alone (Fig. 1B, P = 0.05).

To investigate whether the observed increase in C3 deposition was due to blocking of Crry and not the effect of a higher number of Fc tails or a higher efficiency to activate complement of the mixed IgG1/2a Fc tail, a control bi-mAb directed against the MG42a and CC52 antigens was developed. CC52 is an antigen expressed on CC531 cells, with expression levels comparable with Crry (18). Opsonization of CC531 with MG42a*CC52 resulted in comparable amounts of C3 deposition as opsonization with MG42a (Fig. 1B), indicating that the high level of C3 deposition induced by MG42a*5I2 was indeed due to the inhibition of Crry. In addition to its functional capacity, bi-isotypicity and purity of the bi-isotypic mAb was confirmed by SDS-PAGE (Fig. 1C).

Effect of Modulating mCRP on Outgrowth of Lung Tumors. Subsequently, the effect of modulating Crry on mAb-mediated immunotherapy was investigated in a syngeneic rat colorectal cancer lung metastases model. CC531 cells were preincubated with MG42a-bi-mAb MG42a*5I2, or MG42a-CVF conjugates and injected i.v. in rats (Fig. 2). As a reference, PBS-incubated CC531 tumor cells were injected, resulting in outgrowth of 291 tumors on average at the lung surface. Tumor cells preincubated with MG42a resulted in 175 lung surface tumors on average, which was not significantly different from the PBS group (Mann-Whitney, P = 0.18). Blocking Crry with bi-mAb MG42a*5I2 completely blocked tumor outgrowth in 33% of the rats over all experiments. In these animals, no tumors could be found on the lung surface or inside the lungs. In the other animals, only a few tumors were observed (ranging from 1 to 7 lung surface tumors). This was significantly less compared with the outgrowth of MG42a-incubated tumor cells (P = 0.008). Treatment with MG42a-CVF also resulted in a significantly reduced number of tumors at the lung surface (44 on average) compared with MG42a (P = 0.014), although treatment with bi-mAb was significantly more effective (P = 0.013). No visible tumors were found in other organs than the lungs.

Subsequently, the nature of this noteworthy reduction in the number of lung tumors induced by MG42a*5I2 was investigated. It was determined whether the effect of MG42a*5I2 was indeed caused by inhibition of Crry. Blocking Crry with 5I2 F(ab)_2 resulted in outgrowth of 110 tumors on average (Fig. 3), indicating that the MG4-
BLOCKING Cry on tumor cells prevents tumor outgrowth

Fig. 2. Influence of different therapeutic mAb on outgrowth of lung tumors. To investigate the effect of inhibiting or blocking Cry on tumor cells in vivo, tumor cells were opsonized with different therapeutic mAb (X axis) and injected i.v. in syngeneic rats. The number of outgrowing surface lung tumors (Y axis) is a measure for the effectiveness of the therapeutic effect of mAb. Injection of MG42a*5I2 or MG42a-CVF preincubated tumor cells resulted in significantly less surface tumors compared with MG42a (Mann-Whitney; *, P = 0.008, and **, P = 0.014, respectively). The results of a representative experiment are shown. Each bar represents the average number of lung surface tumors of five rats ± SD.

Fig. 3. Effect of control bi-mAb and F(ab)2 on outgrowth of lung tumors. Tumor cells were opsonized with different therapeutic or control mAb (X axis) and injected i.v. in syngeneic rats. The number of outgrowing surface lung tumors (Y axis) is a measure for the effectiveness of the therapeutic effect of mAb. To investigate whether the effect of MG42a*5I2 mAb was due to the inhibition of Cry, the previous experiments were repeated with a control bi-mAb MG42a*CC52, directed against an irrelevant antigen. The effect of this control bi-mAb was comparable with that of MG42a alone, indicating that the observed effect was indeed mediated by Cry. To check whether the observed effect was complement dependent and not an independent effect mediated by mAb binding to Cry, 5I2 F(ab)2 fragments were used, resulting in a decrease in tumors. Control F(ab)2 did not exert this effect, indicating that the effect was not due to the fact that F(ab)2 fragments were used. The results of a representative experiment are shown. Each bar represents the average number of surface lung tumors from five rats ± SD.

Fig. 4. Influence of prophylactic therapy with bi-mAb. Because MG42a*5I2 was most effective in inhibiting outgrowth of metastases compared with MG4-CVF, the effect of this bi-mAb on tumor outgrowth was further investigated. Clinical mAb therapy in patients will probably be most successful in an adjuvant setting (25, 27); for this reason, a prophylactic experiment was performed. Rats received injections of MG42a*5I2 (300 μg/200 pmol), MG42a*CC52 (300 μg/200 pmol), MG42a (150 μg/100 pmol; equal number of MG4-binding sites between bi-mAb and mAb), or PBS 6 h before tumor cell injection and received injections of a similar dose 6 h after tumor cell injection. Control mAbs only binding to Cry [e.g., 5I2 F(ab)2] were not injected, because they were previously shown to evoke severe side effects or death when injected systemically. After 3 weeks, rats were sacrificed, and the number of metastases was counted. Similar results binding part of the bi-mAb is essential for an optimal reduction of the number of metastases. Tumor cells incubated with control CC52 F(ab)2 induced 344 tumors on average, which was significantly more (P = 0.009). The latter number was comparable with the PBS control group (353 tumors on average; P = 0.917), suggesting that inhibition of Cry was also responsible for the observed effect, probably by allowing unhampered activation via the alternative pathway tick-over mechanism. Injection of CC531 cells incubated with both the parental mAbs [MG42a and 5I2 F(ab)2] resulted in outgrowth of 70 surface tumors, a significantly higher number compared with bi-mAb (P = 0.013), implicating a synergistic effect of bi-mAb compared with both of the parental mAbs (Fig. 3).

Furthermore, to control for increased complement activation due to a higher number of Fc tails or more efficient ADCC of MG42a*5I2-opsonized cells, the control bi-mAb MG42a*CC52 was used. The effect of opsonization with MG42a*CC52 on tumor outgrowth was compared with MG42a*5I2 (Fig. 3). The number of tumors observed after treatment with MG42a*5I2 (6 on average in this particular experiment) was significantly less (P = 0.014) than the number of tumors induced by MG42a*CC52-treated tumor cells (244 tumors on average). The latter number was comparable with the number of tumors on the lungs of rats that received injections of MG42a-opsonized cells (228 tumors on average; P = 0.602). These experiments demonstrate that the effect of bi-mAb MG42a*5I2 was indeed due to its inhibitory effect on Cry function.
Fig. 5. Biodistribution and complement consumption of injected mAbs. Rats received injections of with mAb or bi-mAb 6 h before and 6 h after tumor cell injection. At tumor cell injection as well as 12 h after the last mAb injection, blood was drawn, and the presence of mouse IgG was detected by IgG2a sandwich ELISA (A: PBS and MG4 rat or biotipot ELISA (B: MG4*5I2 and MG4*CC52) as described in “Materials and Methods.” The effect of injection of mAb and tumor cells on complement activation was determined by measuring the hemolytic activity of the sera from the treated rats as described in “Materials and Methods.” C, a representative experiment. Binding of bi-mAb to normal tissue was determined with confocal microscopy. Sections were stained with goat antimouse IgG/FITC and investigated by confocal microscopy. Liver endothelium of MG4*5I2-treated rats was shown to be positive for mouse IgG 2 days after injection (D: 50 μm), in contrast to liver endothelium of MG4-injected rats (inset). Data of representative experiments are shown.

were obtained as observed in the preincubation experiments (Fig. 4). Injection of MG4*5I2 (18 versus 353 on average; P = 0.02). MG4 was less effective in this experimental set-up compared with the preincubation experiments. This resulted in comparable numbers of metastases in the MG4 group compared with the PBS-injected group (353 versus 487; P = 0.368). Although MG4*CC52 showed a trend to be more effective than MG4, in preventing outgrowth (175 versus 353 tumors), the difference in the number of surface tumors was not significant (P = 0.053).

Although no adverse effect after prophylactic treatment with MG4*5I2 was observed as has been described for injecting SI2, the Crry-recognizing arm may also bind to normal cells. Therefore, the biodistribution of MG4*5I2 was investigated. Six h after the first injection, at t = 0, no MG4*5I2 could be detected in the serum by ELISA. In contrast, MG4, and low levels of MG4*CC52 (Fig. 5, A and B) were readily detectable. Twelve h after the last mAb injection (t = 18 h), all mAbs could be detected in the serum at comparable levels (Fig. 5, A and B). In addition, binding to host tissues was investigated. Two days after injection, binding of bi-mAb could be demonstrated to liver endothelium (Fig. 5D). No binding was observed on lung, kidney, and skin tissue (data not shown). In the intestine, only infiltrating immune cells were observed to bind MG4*5I2. Also, peripheral blood lymphocytes were shown to be positive for MG4*5I2 by flow cytometry (data not shown). Biodistribution of MG4 has been described previously (11, 18). MG4 was only shown to weakly bind to cells of the epithelial crypts in the intestine. To investigate whether injection of tumor cells in MG4*5I2-injected rats or binding of MG4*5I2 to host tissue caused complement activation, a CH50 of the serum of these rats at t = 0 h and t = 18 h was performed (Fig. 5C). In contrast to rats that received injections of MG4 or MG4*CC52, the hemolytic activity of the serum of MG4*5I2-injected rats was shown to be decreased 18 h after tumor cell injection. These results show that i.p. injection of MG4*5I2 followed by injection of tumor cells results in complement activation, suggesting that the mechanism by which MG4*5I2 eradicates tumor cells is indeed mediated via complement activation and inhibition of Crry.

DISCUSSION

Because of the species restriction of mCRP, it is important to use an animal model syngeneic for mCRP and complement to study the effect of mCRP on tumor outgrowth. The role of mCRP expression on tumor outgrowth in a syngeneic model is only described in a few studies. An increase in tumor outgrowth of human breast cancer cells transfected with rat Crry was observed in nude rats, whereas transfection with rat CD59 did not result in comparable effects, showing the protective effect of Crry expression (12). Similar results were obtained in a study in which syngeneic hepatoma cells, expressing high levels of Crry, were injected in rats. These rats showed a lower survival rate compared with rats that received injections of cells expressing low levels of Crry (13). These data show the important role of expression of Crry on tumor growth in vivo in rats.

Because an important immune effector mechanism of therapeutic anticancer mAb is activation of the complement system, leading to direct lysis and complement-dependent cellular cytotoxicity, expression of mCRP on tumors is likely to inhibit therapeutic effects. This has previously been confirmed in vitro (3, 14, 28). Predominantly mouse xenograft models have been used in the past to evaluate the effect of immunotherapeutic mAb in vivo, leading to an overestima-
tion of the effect (5, 29). Previously, we have described a syngeneic rat colorectal cancer model to study the role of mCRP expression on tumor cells on mAb-mediated immunotherapy (11). In that study, it was shown that Crry was the most important mCRP on CC531 cells when determining complement activation in vitro, induced by MG42α. When MG42α mAbs were injected in rats, bearing established CC531 liver tumors, no C3 deposition could be observed on these tumor cells in vivo (11). This suggests that mCRP indeed inhibit complement activation of therapeutic mAbs in vivo. In the present study, we have expanded these previous observations by showing that Crry on these tumor cells prevents efficient eradication of mAb-opsonized tumor cells in vivo. Therefore it is important to inhibit or overcome the function of Crry in vivo to increase the effect of mAb-mediated immunotherapy. Previously, it has been shown that systemic complement activation after injection of mAb that inhibit Crry led to severe side effects in these rats (13, 30). This observation demonstrated the necessity to inhibit or overwhelm Crry in a tumor-restricted fashion (4). This can be achieved either by using bi-mAb, directed against a tumor antigen and Crry or mAb-CVF conjugates. In vitro, opsonization of either human renal cell carcinoma or colorectal carcinoma cells with the appropriate bi-mAb (G250*anti-CD55 and anti-EpCAM*anti-CD55, respectively) mediated up to four times the amount of C3 deposition on these cells compared with a monospecific antitumor mAb (14, 16). Also, opsonization of colorectal cancer cells or neuroblastoma cells with anti-Ep-CAM-CVF and anti-GD2-CVF conjugates, respectively, resulted in vitro in increased amounts of complement activation compared with the unconjugated mAb (14, 17). In accordance with these in vitro studies, in the present study, it was demonstrated that in vivo inhibition of a major membrane-bound C3 regulatory protein (Crry) on tumor cells using either MG42α-CVF or bi-mAb MG42α*S12 also substantially improved the outcome of immunotherapy compared with single mAb therapy.

The experimental conditions of this proof-of-principle study, using preincubation of the tumor cells, allowed an accurate determination of the effect of bi-mAb on tumor outgrowth. It was demonstrated that preincubation of CC531 cells with MG42α*S12 bi-mAb could prevent tumor outgrowth, in contrast to control bi-mAb directed against MG42α and an irrelevant antigen in this model. However, under these experimental conditions no cross-reactivity of bi-mAb with Crry expressed on normal cells could occur. Because it was previously shown that injection of 1 mg of S12 led to 50% mortality in 4–8 h (13), we also have investigated the effect of prophylactic treatment with MG42α*S12. In contrast to the harmful effects of monospecific anti-Cry mAb or F(ab)2, MG42*5S12 was shown to be safe when injected i.p. in rats. The pharmacokinetics of bi-mAb were slower compared with MG4 parental mAb because bi-mAb binds to Crry on normal cells, in contrast to MG4. Despite binding to endothelium and blood cells, reflected by this undetectable serum concentration at t = 0 h of the bi-mAb, rats did not show any adverse consequences of this treatment, which supports the potential immunotherapeutic applicability of appropriate bi-mAb. The significant decrease in tumor outgrowth indicates that Crry can indeed be inhibited in a tumor-specific fashion with bi-mAb directed against a tumor antigen and a mCRP. We previously observed similar homing capacities and patterns of MG42α*S12 compared with the parental MG42α mAb in a syngeneic solid liver tumor model, which supports an important role for the antitumor arm in accumulating the bi-mAb at the tumor cell surface and suggests potential applicability in a solid tumor model (data not shown; Ref. 11). Injection of MG42α-CVF in the syngeneic lung metastases model did not lead to detectable side effects, although bi-mAbs were more effective in complement activation in vitro and in reducing the number of outgrowing metastases in vivo compared with the mAb-CVF conjugates.

In a clinical setting, it might be expected that an anti-CVF response will be generated (31), which can hamper the therapeutic effect on the long term. This problem may be circumvented by conjugation of a factor I-insensitive active part of C3b to the antitumor mAb (32). Less immunogenicity can, however, be expected of bi-mAb compared with mAb-CVF. These arguments imply that inhibition of mCRP using bi-mAb will be the preferable immunotherapeutic approach. Ideally for clinical use, the tumor antigen-recognizing arm should be of high affinity, and the mCRP blocking arm should be of low affinity to maximize tumor specificity and minimize a systemic cross-reactivity and thus avoid complement-mediated toxicity (15).

In conclusion, with this proof-of-principle study, which is to our knowledge the first in vivo study in a model syngeneic for mCRP and complement investigating the effect of a membrane-bound C3 regulatory protein (Crry) on mAb-mediated immunotherapy, we show that tumor-specific inhibition of Crry with bi-mAb or overwhelming the function of Crry with antitumor mAb-CVF conjugates (albeit to a lesser degree) is much more efficient in tumor eradicaton than conventional mAb immunotherapy in this colorectal cancer lung metastases model. Bi-mAbs, blocking the most important C3 regulatory protein specifically on tumor cells, are a promising approach to increase the clinical outcome of mAb-mediated immunotherapy.

ACKNOWLEDGMENTS

We thank L. A. Trouw and M. M. Koudijjs for assistance with the animal experiments and J. D. H. van Eendenburg for the development of the bi-mAbs.

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Cancer Res 2004;64:4366-4372.