Immunotherapy with Low and High Affinity Monoclonal Antibodies 17-1A and 323/A3 in a Nude Mouse Xenograft Carcinoma Model

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ABSTRACT

The therapeutic effects of the low and high affinity mAbs 17-1A and 323/A3 were investigated in nude mice xenografted with LS 180 human colorectal carcinoma cells. Treatment of mice grafted with dispersed tumor cells, before formation of a tumor nodule, was started 1 day after s.c. injection of tumor cells and consisted of a single i.p. injection of murine 17-1A or 323/A3 mAb. Tumor appearance after a single injection of either 17-1A or 323/A3 was delayed as compared to injection of an irrelevant mAb. Both 17-1A and 323/A3 reduced the tumor growth rate, and both mAbs decreased the total number of mice that eventually developed a tumor. In all experiments, 323/A3 showed consistently better treatment effects on xenografted mice than 17-1A. For treatment of established tumors with mAb 17-1A or 323/A3 therapy was delayed until tumor growth and mean tumor size as compared to the control group injected with irrelevant mAb. One single i.p. injection of 17-1A had no effect on further tumor growth and mean tumor size of this group as compared to the 17-1A treated mice and the control groups. Multiple injections with mAb 17-1A also had no effects on established tumors, in contrast to mAb 323/A3, where serial injections resulted in tumor growth reduction and, eventually, in some mice reduction in tumor size. In summary, we showed that in nude mice mAb 323/A3 (Ka = 2 × 10^9 M^-1) is much more potent than mAb 17-1A (Ka = 5 × 10^7 M^-1) in eradication of non-established tumors and treatment of small established tumors. These results suggest that high affinity mAbs like 323/A3 might dramatically improve the clinical results obtained thus far with the low affinity mAb 17-1A in the adjuvant treatment of surgically resected Dukes C colorectal cancer patients with minimal residual disease.

INTRODUCTION

Immunotherapy based on mAbs still needs major improvements to fulfill initial expectations of its potential to combat tumors. Based on a relatively specific recognition of tumor cells, the ability to reach the tumors via the blood circulation, and the capacity to mediate target cell lysis through the complement system or through Fc receptor-bearing killer cells, mAbs were initially hailed as magic bullets. However, clinical trials with mAbs in patients with advanced solid tumors have only rarely shown a complete response (1, 2). Recently, the efficacy of mAb treatment in a minimal residual disease setting was reported. After surgical eradication of primary colorectal tumors at the Dukes C stage, adjuvant treatment with mAb 17-1A was shown to reduce 5-year mortality by 30% (3). mAbs, in the correct clinical setting with adequate simultaneous stimulation of effectors, may, therefore, still become widely useful.

Possible reasons for the low efficacy in the treatment of solid tumors have been studied, and poor biolocalization of the mAb into the solid tumor appeared to be a crucial factor. Biolocalization is dependent on vascularization as well as on density of the target antigen on the tumor cells (4). The amount of antibody bound has been shown to influence tumor cell lysis by either ADCC3 or CMC (5). The role of mAb affinity in in vivo tumor cell lysis and the amounts of antibody that have to be bound to the target tumor cells to trigger effector mechanisms are poorly studied. In a previous study (6), we showed evidence for the importance of antibody affinity for ADCC- and CMC-mediated kill of tumor cells in vitro by comparison of murine and human-mouse chimeric versions of the two anti-Ep-CAM (7) mAbs 17-1A and 323/A3, respectively. We reported that chimeric 323/A3 mAb in vitro is more effective than the chimeric mAb 17-1A and that chimeric mAbs of the human IgGl isotype show higher ADCC than the murine IgG2a isotype. Additionally, we also showed that in vitro ADCC with 323/A3 is less sensitive to low antigen expression levels on the target cells than ADCC with mAb 17-1A. Here, we present data on mAb 323/A3 and 17-1A with respect to the impact of antibody affinity on biolocalization and on therapeutic effects on tumor cells in vivo.

MATERIALS AND METHODS

Target Tumor Cell Line. LS 180 human colorectal carcinoma cells (American Type Culture Collection CL 187) were maintained at 37°C and 5% CO2 in DMEM containing 10% FCS and antibiotics. Mice. Male BALB/c nu/nu specific pathogen-free mice, 6 weeks of age, were obtained from IFFA Credo (St. Germain-sur-l'Arbresle, France). After arrival, mice were given the opportunity for acclimatization for at least 1 week before starting any experimental procedure. The animals were kept in the animal facilities of the Academic Hospital Leiden in sterilized cages under sterile filter top conditions and handled in a laminar airflow to protect them from infections. Sterile food pellets and acidified water were supplied ad libitum.

Antibodies. The murine IgG2a mAbs 17-1A (8) and 323/A3, an isotype-switch variant of the original IgGl 323/323/A3 mAb (9), have been raised against the human epithelial adhesion molecule Ep-CAM and were used for our therapy studies. mAb 323/A3 (Ka = 2 × 10^9 M^-1) has an approximately 40-fold higher affinity for Ep-CAM than mAb 17-1A (Ka = 5 × 10^7 M^-1), and both antibodies are competing for binding to this molecule (10, 11). mAb 17F5 directed against the MAM-7 antigen, present on renal and breast carcinomas, and mAb C123b against CD4 present on human T cells but not on LS 180 cells served as control mAbs. For the biolocalization experiments, the human-mouse chimeric forms of 17-1A (12) and 323/A3 (6), with human IgGl heavy chain constant domains, were used. The antibodies were purified by protein A-Sepharose and ion-exchange chromatography (Pharmacia Biotech B.V., Woerden, the Netherlands) and were negative for endotoxin activity by LAL tests.

Biodistribution. Tumors in nude BALB/c mice were induced by s.c. injection of 10^5 LS 180 cells. After 10 days, the mice were injected i.v. with 100 µg chimeric 17-1A or 323/A3 mAb. Mice were sacrificed by cervical dislocation at 4, 24, and 72 h after injection of the mAb. Tumors were excised and immediately fixed in cold isopentane. Frozen sections were stained using anti-human Fc mAbs (Dako A/S, Glostrup, Denmark) to detect the presence of

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3 The abbreviations used are: ADCC, antibody-dependent cellular cytotoxicity; CMC, complement-mediated cytotoxicity; Ep-CAM, epithelial cell adhesion molecule.

4 M. P. Velders; C. M. Rhijn van; E. Oskam, I. H. Brains, H. A. M. Bakker, G. J. Fleuren, S. O. Warnaar, and S. V. Litvinov. The impact of antigen density, antibody affinity and LFA-1 mediated interactions on antibody dependent cellular cytotoxicity, submitted for publication.
chimeric mAb in the tumors. Immunohistochemistry was carried out as described before (13).

Therapy of Human LS 180 Xenografts in Nude Mice. Two weeks prior to tumor cell inoculation into the mice, the LS 180 cells were thawed and expanded in T175 tissue culture flasks (Nunc, Roskilde, Denmark). During this expansion phase, cells were detached with trypsin in 0.1% EDTA and split every other day to keep them in a proliferating state. Before injection into mice, the cells, when approximately 50% confluent, were detached and washed four times with sterile PBS to wash out all FCS. After washing, the cells were counted using trypan blue to distinguish living and dead cells. Viability of the cells always exceeded 95%. Subsequently, cells were brought up to a final concentration in 0.2 ml sterile PBS and injected s.c. into the right flank of the mice. After inoculation of the tumor cells, mice were randomly divided, depending on the experiment, into groups of 6, 8, or 10 mice. Within the experiments, the group sizes were identical for each treatment assignment. At the day of treatment, the cages were randomly selected for the different antibody treatments.

Treatment of not established tumors was started 1 day after inoculation of $1 \times 10^6$ tumor cells by i.p. injection of a single dose of 40, 400, or 4000 µg of either 17-1A, 323/A3, or 175F4 mAb. In one of the experiments, a PBS group served as an additional control. The treatment effects were evaluated by noting the day of appearance of a visible tumor and by measuring the mean tumor volume ($mm^3$) in the groups of mice receiving the different treatments. At the end of the study, the tumor-bearing mice were sacrificed by cervical dislocation. However, tumor-free mice were kept alive to evaluate eventual late tumor appearance. The tumor volumes after each measurement were statistically analyzed by the Mann-Whitney test.

For the experiments on established tumors, 3 $\times 10^6$ tumor cells were injected s.c. in 200 µl sterile PBS to obtain simultaneously established tumors in all mice. In one experiment on established tumors, mice received a single i.p. injection of 400 µg 17-1A, 323/A3, or 175F4 mAb on day 5, when the tumor volumes were between 5 and 10 $mm^3$. In another treatment protocol for established tumors, mice received multiple mAb injections of 400 µg mAb i.p. on day 4 and additionally on days 7, 11, 14, and 18. Here the mean tumor size at the start of treatment was 20 $mm^3$. Tumor volumes were measured on the days of injection and were followed for each mouse individually.

RESULTS

Prevention of Tumor Outgrowth with a Single Injection of 40 or 400 µg mAb. The potency of mAbs 17-1A and 323/A3 to eliminate nonestablished, presumably dispersed tumor cells from BALB/c nude mice xenografted s.c. with LS 180 tumor cells was studied by injecting the mice with a single dose of mAb. The day after inoculation of tumor cells, groups of six mice were injected i.p. with different mAbs at several concentrations. Fig. 1 shows the tumor appearance in mice injected with $1 \times 10^6$ LS 180 cells and treated with 40 or 400 µg mAb, respectively. All mice treated with a 40-µg mAb dose showed a measurable tumor on day 8, similar to the control group, that received 400 µg irrelevant mAb 175F4. In the groups treated with 400 µg 17-1A or 323/A3, the first tumors appeared on day 8, but 100% tumor take was delayed 4 days as compared to both the control and the 40-µg mAb-treated mice. Since the tumor take in all groups eventually was 100%, the mean tumor volume of the different treatment groups over a period of 24 days was compared (Fig. 2). Although mice treated with 400 µg 323/A3 or 17-1A had a similar timing of tumor appearance (Fig. 1B), 323/A3-treated mice had smaller tumors at all times as compared to all other groups. This difference became statistically significant from day 19 on. Treatment of tumor-bearing mice with 40 µg 323/A3 or 17-1A had no significant effect on either tumor appearance or mean tumor volume.

Tumor Cell Neutralization with Single Injection of 400 µg or 4 mg mAb. Since no treatment effect was observed in the groups that received 40 µg mAb, the treatment was repeated using higher mAb doses. Mice were divided in seven groups of eight mice and treated i.p. with 400 µg or 4 mg 323/A3 or 17-1A IgG2a. Groups injected with either 400 µg or 4 mg of the irrelevant IgG2a mAb 175F4 or 200 µl PBS served as controls. Although the mice were inoculated with the same number of LS 180 tumor cells as in the previous experiment, in this experiment, initial tumor appearance was delayed in the groups that received a single 323/A3 mAb injection as compared to the control and 17-1A groups, where tumors started to appear on day 5 (Fig. 3). The delayed tumor appearance in the 323/A3 mAb-treated mice with either 400 µg or 4 mg resulted in a significant difference ($P < 0.05$) in mean tumor size of these groups as compared to the control groups (Fig. 4). Tumor appearance and tumor size in the 175F4-treated mice did not differ from that in the PBS-treated mice. Although in the groups treated with mAb 17-1A initial tumor appearance was not delayed as compared to the controls, the number of mice that eventually developed a tumor was significantly lower than in the control groups. Treatment with 323/A3 resulted in 87.5 and 75% tumor-free mice when treated with a single injection of 400 µg and 4 mg mAb, respectively. Upon treatment with 17-1A, 62.5 and 37.5% of the mice remained tumor free after a single dose of 400 µg and 4 mg mAb, respectively. Tumor-free mice stayed tumor free for 120 days,
of 400/xg gave better results than a dose of 4 mg of either 323/A3 or statistically significant differences as compared to the 175F4 control group (P < 0.05). Mean tumor sizes of groups of six mice are expressed in mm*; bars, SD. *, (g 175F4 (D), 40 (¿g 17-1A (0) or 323/A3 (W), or 400 »ig of 17-1A (É) or 323/A3 mAb grow and form a solid tumor for 5 days. At day 5 when i.p. mAb fig mAb. To study treatment of established tumors with a single mAb after which the experiment was terminated. Overall, a single injection of 400 /xg gave better results than a dose of 4 mg of either 323/A3 or 17-1A, both with respect to tumor appearance and to mean tumor size.

**Treatment of Established Tumors with a Single Injection of 400 /xg mAb.** To study treatment of established tumors with a single mAb injection, 3 × 10⁶ s.c. inoculated LS 180 tumor cells were allowed to grow and form a solid tumor for 5 days. At day 5 when i.p. mAb treatment was given, tumor sizes differed between 5 and 10 mm² with a mean of 6 mm². Since three times more tumor cells had been initially inoculated as compared to previous experiments and tumors were rapidly growing, the study had to be terminated after 19 days when some tumors in the control and 17-1A-treated group reached volumes around 2000 mm³. One single injection with 400 /xg 323/A3 reduced the tumor growth in most of the mice, resulting in a statistically significantly (P = 0.02) smaller average tumor size from day 13 (8 days after mAb injection) until the end of the study (Fig. 5A). In the 323/A3 group of mice, the individual tumor sizes varied more than in the control and 17-1A-treated group (data not shown). Mean tumor sizes of mice from the 17-1A-treated group were identical to the control group.

**Treatment of Established Tumors with Multiple Injections of 400 /xg mAb.** Since a single injection with mAb 323/A3 reduced the tumor growth of established tumors, we studied the effect of multiple mAb injections on tumor growth. Therefore, 3 × 10⁶ LS 180 cells were inoculated s.c. in three groups of 10 nude mice. Four days after inoculation, tumors had reached a mean size of 20 mm³. The size of the tumors at the start of therapy was somewhat larger than in the previous experiment on established tumors. Mice received the first i.p. injection of either 400 /xg 175F4, 17-1A, or 323/A3 mAb on day 4 and additional 400 /xg mAb injections on days 7, 11, 14, and 18. This administration schedule was based on the half-lives of mAb 17-1A and 323/A3 (60–70 h), as observed in studies by Kievit et al. (Free University Hospital, Amsterdam, the Netherlands), and the diminished mAb presence in the tumor nodules at 72 h after mAb injection, as mentioned in the biolocalization section of this manuscript (data not shown). Tumors grew very rapidly; however, at day 14, a reduced growth rate of the 323/A3-treated group was observed as compared to the 17-1A and control groups (Fig. 5B). On day 18, in 3 of 10 of the 323/A3-treated mice, clear signs of tumor necrosis were visible in the center of the tumor, surrounded by a rim of viable tumor mass. The necrosis in the tumors was accompanied by a decrease in individual tumor size in these mice. However, the tumors that showed necrosis in the center did not totally disappear. These signs of necrosis were only observed in the 323/A3-treated mice and were absent in the 17-1A and control groups.

**Biolocalization of 323/A3 and 17-1A in LS 180 Xenografts.** A precondition for treatment of tumors with mAbs is that they localize into the tumor. Therefore, we compared the 17-1A and 323/A3 intratumoral distribution after i.v. injection of the chimeric form of these mAbs into mice bearing small s.c. LS 180 tumors. The use of chimeric forms of the mAbs 17-1A and 323/A3 provided the possibility to detect mAb localization in tumor sections without background staining of irrelevant murine antibodies, which occurred when the murine mAbs 17-1A and 323/A3 were used. Two hundred µl PBS containing 3 × 10⁶ tumor cells were inoculated in nude mice, and after 5 days when mean tumor sizes were 50 mm³, 0.1 mg chimeric mAb 17-1A or 323/A3 were injected i.p. The tumors were excised 4, 24, and 72 h after mAb injection. Sections of these tumors were

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scored for mAb influx by immunohistochemical staining. A clear difference was visible between 17-1A and 323/A3 mAb penetration into the tumors (Fig. 6). After 4 h, mAb 17-1A was distributed more homogeneously in the tumors than mAb 323/A3, that was only detectable on tumor cells around the blood vessels (Fig. 6, A and B). However, staining for mAb 323/A3 was much more intense than staining for mAb 17-1A. After 24 h, 17-1A mAb concentration increased homogeneously throughout the tumor nodule. mAb 323/A3 had penetrated deeper into the tumor mass as compared to 4 h after injection (Fig. 6B) at a higher concentration as compared to mAb 17-1A. Less intense staining of some central areas within the tumor, like for 17-1A (Fig. 6C), was also present for mAb 323/A3 (Fig. 6D), although at lower intensity the mAbs were still detectable in the tumor at 72 h after injection. However, the staining pattern and depth of tumor penetration of the mAbs were comparable with staining at 24 h after injection (data not shown).

DISCUSSION

In the present study, we compared treatments of LS 180 human colorectal carcinoma xenografts in nude mice with, respectively, the low affinity mAb 17-1A and the high affinity mAb 323/A3, both directed against the Ep-CAM molecule (7). In the experiment referred to in Fig. 1, single tumor cells were injected s.c. the day before a single mAb injection. Eventually, all mice developed tumors, and the mean tumor size of the group treated with a single injection of 400 µg 17-1A did not significantly differ from the control group. However, the mean tumor size of the group treated once with 400 µg 323/A3 differed significantly from both the 17-1A and control group. This indicates that in this experiment, 323/A3 not only delayed initial tumor take but also reduced the tumor growth.

In a second experiment, a single mAb 323/A3 injection of 400 µg or 4 mg delayed or even prevented tumor take, resulting in a decreased mean tumor size of the specifically treated groups as compared to the group treated with irrelevant mAb. In this experiment, treatment with 17-1A also reduced total tumor take and mean tumor size (P < 0.05) as compared to the control group, however, less than treatment with 323/A3 (P < 0.01). Although differences were observed between 17-1A and 323/A3 at both 400 µg and 4 mg, these differences were not statistically significant. A mAb dose of 4 mg seemed less effective (not statistically significant) than the 400-µg dose, which may be optimal in this model. The existence of an optimal mAb dose for tumor therapy has been suggested by Johansson et al. (14). Additionally, these authors showed that early antibody treatment is crucial to prevent tumor take, since a delay of treatment from day 0 to day 4 in their model increased tumor incidence from 5 to 95% of all mice. This indicates the extremely low efficiency of solid tumor eradication in a xenograft model. Their observation that a treatment delay of 1 day has a significant impact on total tumor take suggests that minor differences in tumor size at the start of the treatment do have an important effect on the final outcome of the therapy. In this study, 100% tumor take in the control groups of the first (Fig. 1) and second experiment (Fig. 3) was achieved on day 8 and around day 20, respectively. Differences in the number of injected cells and the aggressiveness of these cells, as a result of density in the culture, may account for the differences in tumor appearance between our first and second experiment. Additionally, differences in the number of effector cells between mice of different breeds may lead to the differences observed.

Apparently, there is a maximal tumor load that can be effectively treated with mAbs. This is also illustrated by our treatment of established tumors with single or multiple injections of 400 µg 323/A3 or 17-1A. Treatment with either a single or with multiple 17-1A mAb injections had no effect on the mean tumor size, whereas treatment with 323/A3 reduced the overall tumor size by approximately 50%. Although mAb 17-1A could eradicate single tumor cells from the...
mice, the tumor load of established tumors was apparently too high for mAb 17-1A to have any significant effect on the mean tumor size. mAb 323/A3, in contrast, significantly reduced the growth of established tumors. This illustrates that the impact of tumor size at the time of first treatment on the final outcome of the treatment is dependent on the affinity of the mAb used. The results of the 17-1A treatment presented in this study are in agreement with the reported efficacy in the treatment of minimal residual disease (3) and the lack of efficacy observed in advanced tumors (15, 16). Tumor necrosis as observed in a few mice treated with multiple 323/A3 injections suggests a stronger effect on the tumor than a single mAb injection. To conclude whether multiple injections of 323/A3 against established tumors are superior over a single mAb injection, a study should be carried out in which the different treatments are started simultaneously in mice that bear equal-size tumors.

The use of model systems to study treatment of established tumors with mAbs is not optimal. For efficient tumor take in our model, the mice were inoculated with high cell numbers of an aggressively growing tumor that, within 3 weeks, expanded from 0 to approximately 2 g (1/10 of the total weight of the mice). This is clearly different from mean tumor growth rates in patients. The high growth rate may overwhelm the host defense system, such as ADCC and CMC. The use of nude mice, necessary to avoid rejection of the human cells, does not permit the interaction of the injected antibodies with an intact immune system. For example, no antidiotypic antibody response can be expected, which might be correlated with the clinical outcome (17). Finally, the eradication of human tumor cells from the mouse by ADCC requires an interaction with murine effector cells.

We have evidence that ADCC in vitro between target and effectors from different species is less efficient than between species-matched cells, suggesting that eradication of human xenografts from mice is biased by species differences.

The mechanism of tumor eradication mediated by unconjugated mAb in vivo is supposed to be mainly ADCC, because macrophage and natural killer cell depletion resulted in a 100% increase in tumor take in mAb-treated xenografts in nude mice (18). Many groups have studied Fc receptor-expressing cells in the tumor nodules and have isolated such cells including macrophages, monocytes, and NK cells. These cells were capable of killing tumor cells by ADCC (19). Another possible mechanism of cytotoxicity in a xenograft model is complement activation. Autologous cells usually will not be killed by complement since they express complement down-regulating factors (20, 21). However, xenografted human cells lack inhibitors of murine complement, which would allow CMC upon treatment with murine mAbs in the mouse. Despite the possibility of CMC as cytotoxic mechanism, ADCC seems to be the major cytotoxic tool of the mouse. Autologous murine B16 melanoma cells that express murine complement inhibitors that prevent CMC, when grafted in C57BL/6 mice, were eradicated in an antibody-mediated fashion (22, 23). This lysis was shown to correlate with the activation of ADCC-mediating cells. Moreover, Herlyn and Koprowski (18) showed that complement-depleted mice, in contrast with macrophage-depleted mice, could eradicate human tumor xenografts upon mAb treatment as efficiently as noncomplement-depleted mice (18). These results suggest that in the treatment of human tumors with mAbs, CMC can be neglected and that ADCC is the major effector mechanism.
The effect of affinity on mAb tumor penetration and accumulation is important in immunotherapy with unconjugated mAbs. We examined this for mAbs 323/A3 and 17-1A, which compete for binding of Ep-CAM (10, 11), by injecting their chimeric forms into tumor-bearing mice. Twenty-four h after injection, 323/A3 was detectable in the tumor at high concentrations with a gradient from the perivascular space deeper into the tumor, whereas 17-1A showed a much more diffuse but homogeneous staining of the tumors. These results are in agreement with in vitro results of Langmuir et al.,5 who studied penetration of radiolabeled 17-1A and 323/A3 in human multicell spheroids. Despite the different intratumoral distribution pattern, they determined that 323/A3 accumulated in the multicell spheroids to 8–10-fold higher concentrations and showed a superior tumor:blood ratio as compared to 17-1A. Thus, high affinity antibodies may irreversibly bind to the first antigen molecules they reach and thus be prevented from diffusion into other parts of the tumor nodule. This has been described as a “binding site barrier” specific for high affinity mAbs (11, 24). Based on our combined treatment results, we suggest that the local high concentrations obtained in the tumor with the high affinity 323/A3 mAb may be crucial for effective tumor eradication. The relatively low amounts of 17-1A accumulating in the tumor and its diffuse distribution may be the main reason for the absence of therapeutic results in mice bearing established tumors, because the mAb concentration at the cell surface may be insufficient to trigger ADCC (6).

In summary, the in vivo results presented show differences in treatment of human tumor xenografts in nude mice with high affinity mAb 323/A3 and the low affinity mAb 17-1A, in favor of 323/A3. However, these differences were not as pronounced, as suggested by our in vitro data, where ADCC and CMC with 323/A3 was 100- to 1000-fold more effective than with 17-1A (6). The in vivo data presented in this paper confirm our previous in vitro data that 323/A3 may be a better antibody than 17-1A for the treatment of minimal residual disease and may additionally be effective in patients with limited numbers of small macroscopic tumors.

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