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CpG-A and B Oligodeoxynucleotides Enhance the Efficacy of Antibody Therapy by Activating Different Effector Cell Populations[1]

Heidi H. van Ojik, Lisette Bevaart, Christopher E. Dahle, Annie Bakker, Marco J. H. Jansen, Martine J. van Vugt, Jan G. J. van de Winkel, and George J. Weiner[2]

Department of Medical Oncology, Erasmus MC–Daniel den Hoed Cancer Center, 3000 DR Rotterdam, the Netherlands [H. H. v. O.]; Immunotherapy Laboratory and Department of Immunology, University Medical Center Utrecht, 3584 EA Utrecht, the Netherlands [H. H. v. O., L. B., A. B., M. J. H. J., M. J. v. V., J. G. J. v. d. W.]; Genmab, 3584 CK Utrecht, the Netherlands [M. J. v. V., J. G. J. v. d. W.]; and The Holden Comprehensive Cancer Center and Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242 [C. E. D., G. J. W.]

ABSTRACT

Immunostimulatory CpG oligodeoxynucleotides (ODNs) can enhance the therapeutic effect of monoclonal antibodies (mAbs) by enhancing antibody-dependent cell-mediated cytotoxicity (ADCC). Distinct classes of CpG ODNs have been found recently to stimulate different effector cell populations. We used murine cancer models to explore the role of various effector cell populations in the antitumor activity seen with mAbs combined with CpG ODNs of the A and B classes. In the 38C13 syngeneic murine lymphoma model, both CpG A and CpG B enhanced the efficacy of murine antilymphoma mAb. Depletion of natural killer (NK) cells alone markedly decreased the efficacy of therapy with mAbs plus CpG A. In contrast, depletion of both NK cells and granulocytes was required to decrease the efficacy of mAb plus CpG B. A human (h) Fc γ receptor I (FcγRI)-expressing transgenic (Tg) mouse model was used to explore the role of FcγRI in therapy with mAb and CpG ODN. CpG B induced up-regulation of FcγRI in hFcγRI Tg mice, whereas CpG A did not. *In vitro* CpG B also enhanced ADCC of HER-2/*neu*-expressing tumor cells by the FcγRI-directed bispecific antibody MDX-H210 using hFcγRI-positive effector cells. In a solid tumor model, tumor growth was inhibited in Tg mice treated with a combination of MDX-H210 and CpG B. These data suggest that CpG A enhance ADCC largely by activating NK cells. In contrast, other effector cell populations, including granulocytes, contribute to the antitumor activity of CpG B and mAbs. FcγRI plays an important role in this activity.

INTRODUCTION

mAbs[3] have been a valuable addition to current cancer treatment modalities. Recent experience with mAbs such as rituximab and trastuzumab demonstrate that these drugs are well-tolerated and capable of initiating tumor regression in a significant percentage of patients (1, 2). Unfortunately, the majority of patients treated with mAbs exhibit only short-lived partial responses. Our current understanding of the mode of action of therapeutic mAbs is incomplete (3). A variety of mechanisms may be important including blocking of activation signals, induction of growth arrest, induction of apoptosis, complement mediated lysis, and ADCC (4–6). A greater understanding of the mechanisms underlying mAb-induced antitumor activity is essential if we are to develop rationally designed immunotherapeutic protocols that improve on current clinical results. Ab dependent cell-mediated cytotoxicity mediated by FcRs appears to be critical for many mAb-induced antitumor effects (7, 8). Immunotherapeutic approaches have mainly concentrated on leukocyte FcR for IgG (FcγR; Ref. 9). Three classes of FcγR are currently recognized: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16; Refs. 10, 11). FcγRI represents the only receptor class capable of binding monomeric IgG. It has a limited cell distribution and exhibits potent immune-activating activities. Because of these characteristics, this receptor has been considered an optimal trigger molecule for Ab therapy (12). Although granulocytes are not often considered primary mediators of ADCC, they are among the most common leukocytes, can be induced to express FcγRI (13), and can mediate ADCC via FcγRI (14).

Whereas the acceptance of cancer immunotherapy as part of standard clinical practice is relatively new, immunotherapy of cancer is an old concept. At the end of the 19th century, Coley (15, 16) observed therapeutic effects in cancer patients treated with a crude bacterial extract. At that time, the role of the immune system in combating disease was poorly understood, and Coley's results were neither reproducible nor understood from a mechanistic point of view. The past 20 years have seen striking advances in the field of immunology. Some of these advances reflect back on Coley's observations. In particular, we now know that specific sequences within bacterial DNA contain unmethylated CpG dinucleotides that are potent immunostimulatory motifs (17, 18). Synthetic ODNs containing such unmethylated CpG motifs mediate similar effects (19). In particular, CpG ODNs are able to activate immune effector cells and enhance cytotoxicity against tumor targets (20). In animal models, CpG ODNs have potent antitumor effects when administered *in vivo* either alone or in combination with mAb (21–23). Interestingly, the effect of CpG ODNs on various effector cells varies depending on the sequence of the CpG ODNs (20, 24). CpG ODNs with a chimeric backbone in combination with poly-G tails are known as CpG A (also known as "D" type ODNs; Ref. 24). These CpG ODNs are potent inducers of IFN-α production and NK lytic activity, but have little effect on secretion of Th1-type cytokines or B-cell proliferation. In contrast, CpG B (also known as "K" type ODNs) are potent activators of B cells and induce production of Th1-type cytokines. CpG A can induce regression of established NK-sensitive melanomas in mice (22) whereas CpG B does not. In contrast, CpG B is effective at inducing regression of the EL4 murine lymphoma where both NK cells and T cells contribute to the antitumor effect. CpG A is not as active in this model.

Use of the combination of Abs and immunostimulatory agents, such as CpG ODNs, should allow us to combine the specificity of Abs with the potency of the innate immune system. However, doing so in a rational fashion requires an understanding of the potential interaction between these different but interactive arms of the immune system. In the murine studies outlined below, we explore the cellular effectors responsible for the antitumor effects of mAbs when administered either alone or in combination with immunostimulatory CpG ODNs of the A or B class. The results of these studies demonstrate that different types of effector cells can contribute to ADCC, and point to the

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[2] To whom requests for reprints should be addressed, at Holden Comprehensive Cancer Center, 5970Z JPP University of Iowa, 200 Hawkins Drive, Iowa City, IA 52242. Phone: (319) 353-8620; Fax: (319) 353-8988; E-mail: george-weiner@uiowa.edu.

[3] The abbreviations used are: mAb, monoclonal antibody; Ab, antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; BsAb, bispecific antibody; DC, dendritic cell; FACS, fluorescence-activated cell sorter; FcR, Fc receptor; FcγR, receptor for Fc domain of IgG; h, human; mG-CSF, murine granulocyte colony-stimulating factor; NK, natural killer; NTg, nontransgenic; ODN, oligodeoxynucleotide; Tg, transgenic; TLR9, toll-like receptor 9.

potentially potent activity of granulocytes and other FcγRI-expressing cells in the antitumor effect of mAbs alone and Abs plus CpG B *in vivo*. Perhaps most importantly, they highlight the concept that a growing understanding of the cellular mechanisms responsible for the antitumor effects of mAbs should allow us to develop rationally designed combination immunotherapeutic approaches.

MATERIALS AND METHODS

Tumor Cell Lines. The T3C variant of the 38C13 mouse lymphoma cell line (Ref. 25; henceforth referred to as 38C13) were cultured in medium consisting of RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 50 μg/ml streptomycin, 50 IU/ml penicillin, and 4 mM L-glutamine (complete medium) supplemented with 2-mercaptoethanol. SK-BR-3, a human breast carcinoma cell line overexpressing HER-2/*neu*, was obtained from the American Type Culture Collection (Manassas, VA; HTB-30; Ref. 26). The 3-methylcholantrene-induced mouse fibrosarcoma cell line, CMS7HE, stably transfected with human HER-2/*neu*, together with a control cell line, transfected with an empty vector, CMS7neo, were kindly provided by Dr. Hiroshi Shiku (Mie University School of Medicine, Mie, Japan; Refs. 27, 28). These cells were maintained in complete medium, and in case of the CMS7 cell lines supplemented with 462 μg/ml Geneticin (G418 sulfate; Life Technologies, Inc.). All of the adherent cell lines were detached by using trypsin-EDTA (Life Technologies, Inc.) in PBS. Cells used for tumor inoculation were collected in log-phase, and were tested for stable HER-2/*neu* expression by FACS analyses, and for potential *Mycoplasma* contamination before each experiment.

CpG ODN. CpG ODN 1585 (sequence GGGGTCAACGTTGAGGGGGG; Ref. 22) and CpG ODN 1826 (TCCATGACGTTCCTGACGTT; Ref. 29) were used as prototypic CpG A and CpG B, respectively, as we have done previously (22). ODN 1982 (TCCAGGACTTCTCTCAGGTT) was used as a control sequence. All of the ODNs were supplied by Coley Pharmaceutical Group (Wellesley, MA). CpG ODNs were tested and proved to contain <12.5 ng/mg of lipopolysaccharides levels by *Limulus* assays (LAL-assay; BioWhittaker, Walkersville, MD). ODNs were injected i.p. at a dose of 100 μg in 0.2 ml PBS unless otherwise stated.

Flow Cytometry. Whole mouse blood, WBCs, or tumor cells were incubated for 30 min with either labeled or unlabeled mAb. Labeled cells were washed three times in PBS supplemented with 1% bovine serum albumen and 0.01% azide. When unlabeled primary mAbs were used, indirect staining, using FITC- or r-phycoerythrin-labeled $F(ab')_2$ fragments of a goat α-mouse mAb as a labeled second Ab was performed. Whole blood samples were lysed and fixed by using FACS lysing solution (Becton Dickinson, San Jose, CA). All of the samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). A panel of antimouse mAb, labeled with either FITC or r-phycoerythrin, was used to detect the different mouse effector cell populations. Unconjugated murine mAbs against HER-2/*neu* (520C9; mouse IgG1) were provided by Medarex (Medarex Inc., Annandale, NJ).

ADCC Assay. A ^{51}Cr release assay, slightly modified from Valerius *et al.* (13) was used. Mouse whole blood was used as the source of effector cells. Mice were treated for 3 days with 150 μg of mG-CSF s.c., kindly provide by Dr. Jeff Andresen (Amgen, Thousand Oaks, CA), to increase circulating numbers of leukocytes before blood collection via orbital puncture. Briefly, tumor cells were incubated with 200 μCi ^{51}Cr for 2 h. After washing three times with culture medium, 5×10^3 target cells were added to round-bottomed microtiter plates containing 50 μl of whole blood, CpG ODNs, and sensitizing mAbs. The final volume was 200 μl, and the E:T cell ratio was ~80:1. After incubation overnight at 37°C, assays were stopped by centrifugation. ^{51}Cr-release was measured in supernatants from triplicate wells. Percentage of cellular cytotoxity was calculated using the formula:

$$\%\ \text{specific lysis} = \frac{\text{experimental cpm} - \text{basal cpm}}{\text{maximal cpm} - \text{basal cpm}} \times 100$$

with maximal ^{51}Cr release determined by adding Zap-oglobin (Coulter Electronics LTD, Luton, England; 10% final concentration) to target cells, and basal release measured in the absence of sensitizing mAb and effector cells. Only very low levels of Ab-mediated noncellular cytotoxicity (without effector cells) and Ab-independent killing were observed under these conditions (<5% specific lysis).

38C13 Murine Lymphoma Model. The 38C13 murine lymphoma tumor model has been described previously (30–32). Female C3H or C6B3F1 (C3H × C57Bl6 F1) mice were purchased from Harlan Biosciences, (Indianapolis, IN), housed in the University of Iowa Animal Care Facility, and used when they were 6–9 weeks old. For *in vivo* experiments, 5×10^3 cells growing in log phase were injected i.p. into immunocompetent mice. Day 0 was defined as the day of tumor inoculation. The therapeutic murine mAb MS11G6 (IgG2a) and MS5A10 (IgG1; Ref. 25) were obtained from tissue culture supernatant by protein A (MS11G6) or protein G (MS5A10) affinity chromatography. Mice were treated with doses of 0.1 mg therapeutic mAb in 0.2 ml PBS i.p. as indicated. The University of Iowa or University of Utrecht animal care and use committees approved all of the mice experiments.

NK and Granulocyte Depletion. NK cells were depleted *in vivo* by i.p. injection of 0.1 mg of the anti-NK1.1 mAb PK136 (ATCC HB191) on days −2, 0, 3, 5, 7, and 10. C6B3F1 mice were used for deletion studies, because NK cells from C3H/HeN mice do not express the NK1.1 antigen. Preliminary studies demonstrated that NK cells from C6B3F1 express NK1.1 and are depleted after PK136 therapy as determined by flow cytometry and functional assays for NK activity (lysis of YAC-1 cells). In addition, the *in vivo* development of lymphoma and survival after inoculation with 38C13 cells in C6B3F1 mice is indistinguishable from that in homozygous mice C3H/HeN mice, and the antilymphoma effect of MS11G6 is similar in homozygous C3H/HeN and C6B3F1 mice (data not shown). Granulocytes were depleted by i.p. injection of 0.1 mg of mAb RB6-8C5 (Anti Ly-6G; Kindly supplied by Dr. J. Harty, University of Iowa) on days −2, 3, 5, and 10. Depletion of granulocytes from the peripheral blood for up to 10 days after tumor inoculation was confirmed by examination of peripheral blood smears.

Human FcγRI Tg Mice. FVB/N mice Tg for hFcγRI (CD64) were crossed back into BALB/c (33, 34). In all of the experiments with hFcγRI animals, the Tg mice were matched with their NTg littermates. Mice were bred and maintained in the Tg Mouse Facility of the Central Laboratory Animal Facility (Utrecht, the Netherlands) and were used at 8–16 weeks of age.

Solid Tumor Model. Tumor cells were tested for stable HER-2/*neu* expression after *in vivo* passage by FACS analyses. CMS7HE cells (2×10^6) were injected s.c. in the right flank of male F12 Tg-hFcγRI and NTg mice (27). These tumors grew quickly and could easily be measured using calipers. Tumor diameter was reported as length × width × height (mm^3). BsAb MDX-H210 (hFcγRIa × HER-2/*neu*), produced by chemically cross-linking F(ab′) fragments of the target Abs H22 (hFcγRI), and 520C9 (HER-2/*neu*) was supplied by Medarex (35). MDX-H210 was injected i.p. and CpG ODN s.c. in the vicinity of the tumor as indicated. Mice were checked three times a week for signs of toxicity and discomfort, including level of activity, ruffled fur, diarrhea, and general appearance.

Statistical Analysis. Group data were reported as mean ± SE. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student's *t* tests. Levels of significance are indicated, with significance accepted at the $P < 0.05$ level. Statistical analyses on survival curves were performed using Prism 3.02 software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Both CpG A and CpG B Enhance the Efficacy of mAb Therapy. As outlined above, we have found monotherapy with CpG A (1585) to be more effective than CpG B (1826) in the treatment of NK-sensitive malignancy, whereas CpG B is more effective in models where T cells were found to be responsible for much of the antitumor activity (22). We also demonstrated previously that the efficacy of antitumor IgG2a is significantly enhanced by cotherapy with CpG B (29, 36). If NK cells play the central role in the enhanced ADCC induced by CpG ODNs, we would expect CpG A to be more effective than CpG B at enhancing the efficacy of mAb therapy, whereas CpG B would be more effective if most of the antitumor activity is mediated by other effector cells. Both CpG A and CpG B enhanced the efficacy of IgG2a mAb therapy to a similar degree under a broad

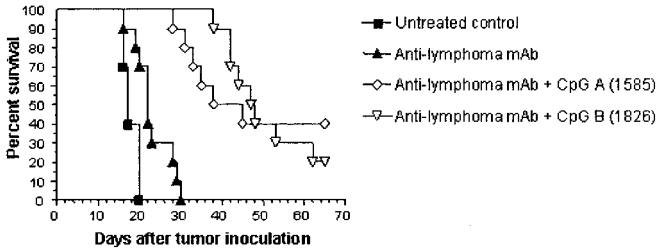

Fig. 1. Both CpG A and CpG B can enhance the efficacy of therapy with antilymphoma IgG2a mAb. Mice were inoculated i.p. with 38C13 tumor cells on day 0, and treated with CpG ODN and mAb on days 5, 7, and 10 with 100 μg anti-lymphoma IgG2a mAb (*MS11G6*) alone or with 20 μg of CpG ODN 1585 (CpG A) or CpG ODN 1826 (CpG B). Survival was recorded daily. Similar results were found in three independent experiments.

range of conditions including studies where therapy was given once early in tumor development (data not shown) and studies of delayed therapy using multiple doses of mAb and CpG ODN (Fig. 1). This suggests that both CpG A and CpG B can activate effector cell populations capable of mediating ADCC.

Antitumor Activity of IgG1 and IgG2a mAb in Combination with CpG ODN. When used as a single agent, neither CpG A nor CpG B had a detectable antitumor effect in the 38C13 lymphoma model *in vitro* or *in vivo*. However, this does not exclude the possibility that the synergy between mAb and CpG ODN is because of direct effects on the malignant cells of the combination of mAb and CpG ODN. We previously produced and evaluated a panel of syngeneic, anti-38C13 mAbs with identical specificity but varied isotypes (25). mAbs of the murine IgG2a isotype have Fc that react with both FcγRIII (CD16) and FcγRI (CD64), whereas mAb of the murine IgG1 isotype have lower affinity for FcγRI (37), particularly when the mAb is in its monomeric form. Thus, one would expect the antitumor IgG2a mAb to be capable of mediating ADCC via a variety of effector cell types, whereas the antitumor IgG1 of the same specificity would signal as well, but would not mediate ADCC as well. Therefore, we evaluated therapy of tumor-bearing mice with antitumor IgG1 combined with CpG A or CpG B. Neither CpG A nor CpG B had a detectable effect on the efficacy of antitumor IgG1 mAb using conditions where both CpG A and CpG B enhance the efficacy of IgG2a mAb (Table 1). In addition, antitumor F(ab′)2 had no detectable antitumor activity either alone or combined with CpG ODN. These studies indicate that the enhanced antitumor effect of mAb plus CpG ODN requires interaction with an effector cell population that has receptors for IgG2a, and supplies additional evidence that enhanced ADCC, and not signaling on the tumor cell, is responsible for the observed antitumor effects in this model.

NK Cells Are Responsible for the Efficacy of CpG A Plus mAb, Whereas a Variety of Effector Cells Contribute to the Efficacy of CpG B Plus mAb. Additional studies were done to explore the role of NK cells in the antitumor effect of IgG2a mAb when used alone or in combination with either CpG A or CpG B. Depletion of NK cells markedly decreased the efficacy of mAb plus CpG A. In contrast, depletion of NK cells with anti-NK1.1 alone had little effect on the antitumor activity of mAb alone (Table 1) or mAb plus CpG B (Fig. 2*B*). This finding is consistent with prior studies that suggest the major effector cell activated by CpG A is the NK cell. In contrast, CpG B activates a variety of cell populations that could mediate enhanced ADCC in the absence of NK cells.

Depletion of Both Granulocytes and NK Cells Decreases the Therapeutic mAb Efficacy of CpG B Plus mAb. Activated granulocytes can express FcγRI and can mediate ADCC (38). Therefore, we evaluated the importance of granulocytes, both alone and in combination with NK cells, in mediating the antitumor activity of mAb and CpG B in the 38C13 lymphoma model. Depletion of granulocytes alone had little impact on efficacy of mAb alone (Table 1) and mAb plus CpG B (Fig. 3). However, depletion of both NK cells and granulocytes decreased the efficacy of therapy under both of these conditions. Additional studies were done to confirm that this decreased survival in NK and granulocyte-depleted mice was because of changes in antilymphoma activity of mAb, and not to other immunological changes induced by effector cell depletion. Postmortem examination of select mice demonstrated that mice died of widespread lymphoma and not infection. Depletion of NK cells and granulocytes had no detectable impact on the behavior or survival of tumor-free mice or on the growth of tumor in mice that were not treated with mAb (data not shown). We also explored the pharmacokinetics of antilymphoma mAb to confirm that the procedures used to deplete NK cells or granulocytes did not impact on clearance of the therapeutic mAb. Depletion had no effect on the levels of the antilymphoma mAb (data not shown).

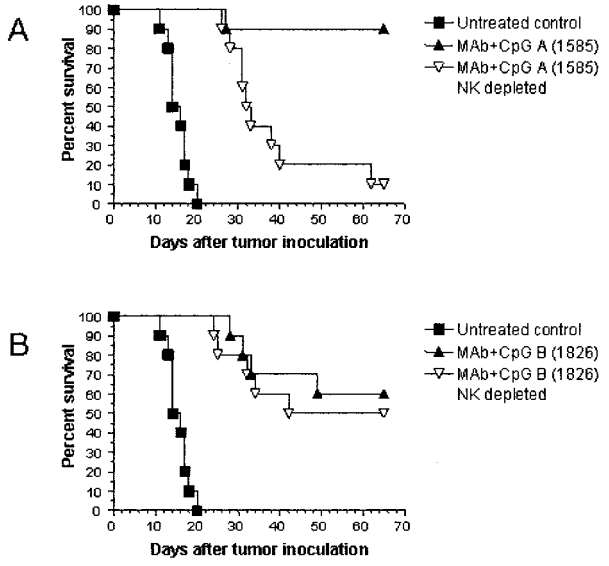

Fig. 2. Depletion of NK cells decreases the efficacy of mAb plus CpG A, but has no effect on the efficacy of mAb plus CpG B. Mice were inoculated i.p. with 38C13 T3C tumor cells on day 0. They were treated with a single dose of 100 μg MS11G6 and 100 μg CpG ODN on day 3. NK cells were depleted by i.p injection of Anti NK1.1 mAb (*PK136*) on days −3, 0, 3, 6, 10, and 13. *A*, mice treated with mAb plus CpG A. *B*, mice treated with mAb plus CpG B. Similar results were found in three independent experiments.

Table 1 *Summary of in vitro and in vivo effects of CpG A and CpG B*

	No ODN	CpG A	CpG B
Enhances therapeutic effect of antitumor IgG2a	−	Yes	Yes
Enhances therapeutic effect of antitumor IgG1 or F(ab′)2	−	No	No
NK depletion decreases efficacy with mAb therapy +/− ODN	No	Yes	No
Granulocyte depletion decreases efficacy with mAb therapy +/− ODN	No	No	No
NK and granulocyte depletion decreases efficacy with mAb therapy +/− ODN	Yes	Yes	Yes
Induces upregulation of hFcγRI	−	No	Yes
Enhances anti-tumor effect of anti-hFcγRI × anti-HER-2/*neu* BsAb	−	Not tested	Yes

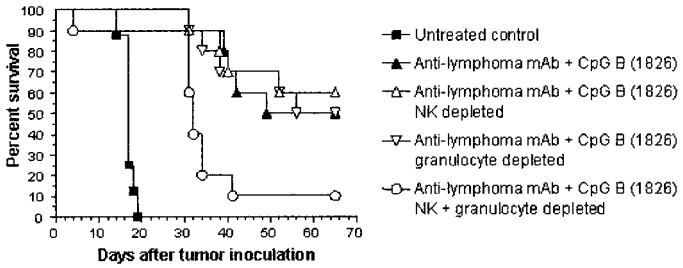

Fig. 3. Depletion of both granulocytes and NK cells decreases the efficacy of therapy with mAb plus CpG B. *In vivo* studies were done to assess whether depletion of granulocytes impacts on the efficacy of mAb therapy plus CpG B. NK cells and granulocytes were depleted using anti-NK1.1 mAb (*PK136*) or anti Ly-6G mAb (*RB6-8C5*), respectively, as outlined in "Materials and Methods." Treatment was given as outlined in Fig. 2. Survival was recorded daily. Similar results were found in three independent experiments.

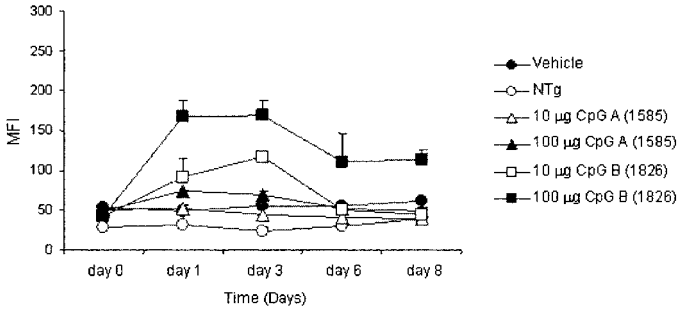

Fig. 4. *In vivo* therapy with CpG B but not CpG A induces up-regulation of hFcγRI by granulocytes. Human FcγRI-Tg mice and NTg littermates serving as controls were injected s.c. with 100 μl vehicle (PBS) or a single dose of 10 or 100 μg CpG ODN A or CpG ODN B. Human FcγRI expression on granulocytes was determined by FACS analysis between 0 and 8 days after CpG ODN therapy. All determinations were performed in triplicate. Results represent mean from two independent experiments; *bars,* ±SD.

Human FcγRI is Up-Regulated by CpG B. The data outlined above suggest effector cells that express FcγRI mediate the enhanced antitumor activity observed when CpG B is added to mAb. Furthermore, granulocytes can play an important role in the antitumor effects observed with this therapy. Therefore, we evaluated the effect of CpG ODN on FcγRI expression. Because Abs against murine FcγRI were not yet available, we used an hFcγRI Tg mouse model to assess FcγRI expression and function. This model has been described previously (33). Tg hFcγRI mice express hFcγRI under control of the endogenous human FcγRI promotor. Human FcγRI Tg mice constitutively express hFcγRI on monocytes, macrophages, immature DCs, and in low numbers on resting granulocytes (34) as would expected for FcγRI. In addition, expression of hFcγRI on granulocytes in this model is up-regulated *in vivo* upon stimulation with IFN-γ or granulocyte colony-stimulating factor (34, 39, 40). Human FcγRI expression was determined at various time points after a single s.c. dose of CpG ODN. As illustrated in Fig. 4, enhanced hFcγRI expression by granulocytes was seen in Tg mice treated *in vivo* with CpG B. In contrast, little if any change in hFcγRI expression was seen with CpG A, even at a high dose. No hFcγRI was detected in NTg mice. A clear time-response curve is seen with hFcγRI expression after a single s.c. dose of CpG B, with hFcγRI expression up-regulated for >8 days after a single 100 μg dose of CpG B. Treatment with CpG B, but not CpG A, enhanced hFcγRI expression levels on monocytes and DCs as well in a dose-dependent manner (data not shown).

Tumor Cell Killing *in Vitro* Mediated by hFcγRI Is Enhanced by CpG B. Next, we investigated whether CpG B had an impact on ADCC. This was done using the hFcγRI-directed BsAb MDX-H210. Effector cells for these assays were harvested by obtaining whole blood from mG-CSF-treated mice and incubating the blood *in vitro* with CpG B. Tg granulocytes activated with CpG B exhibited enhanced MDX-H210, mediated cell killing, with the combination of MDX-H210 and CpG B being effective at very low MDX-H210 concentrations (Fig. 5). No specific lysis was observed with a control CpG B. Non-Tg granulocytes were unable to mediate lysis, except via mAb 520C9, an anti-HER-2/*neu* mouse IgG1 mAb that initiates cytotoxicity via mFcγRII/III (41).

Therapy with hFcγRI × HER/2-*neu* BsAb and CpG B Inhibits Tumor Growth. Additional studies were done to evaluate the *in vivo* effect of CpG B and BsAb using hFcγRI Tg mice, MDX-H210, and mouse fibrosarcoma cells that express the HER-2/*neu* target antigen (CMS7HE). *In vitro* studies indicated that CpG B had no detectable effect on tumor cell morphology, proliferation, antigen expression, or viability (data not shown). Clear reduction in tumor growth was observed in Tg mice treated with the combination of MDX-H210 and CpG B, whereas tumors grew progressively in all of the other treatment groups (Fig. 6), and in control ODN (1982)-treated and NTg mice (data not shown). Treatment in Tg mice with the MDX-H210 alone did not induce an antitumor effect. This is most probably because of inadequate expression of FcγRI by nonstimulated effector cells, thereby limiting FcγRI-mediated targeting and cytotoxicity. Paradoxically, tumors in animals treated with BsAb alone appeared to grow faster than tumors in control mice; however, this difference was not statistically significant.

DISCUSSION

mAbs are now an accepted component of lymphoma and breast cancer therapy. Nevertheless, there is still much that we do not understand about their mechanism of action. Most investigators agree that ADCC plays a major role in the observed responses. Two populations of effector cells (NK cells and monocytes/macrophages) have received the most attention as mediators of ADCC.

Given that CpG ODNs activate NK cells, monocytes, and macrophages, and induce production of immunostimulatory cytokines, it is rational to explore how CpG ODN impacts on the efficacy of mAb therapy, and which effector cells contribute to this antitumor activity. We used the differential effects of CpG A and CpG B to assess which effector cells contribute to the antitumor effect of therapy with mAb plus CpG ODN. CpG A (1585) and CpG B (1826) enhanced the efficacy of mAb to a similar degree. However, this enhanced effect was mediated by different effector cell populations. For CpG A, NK

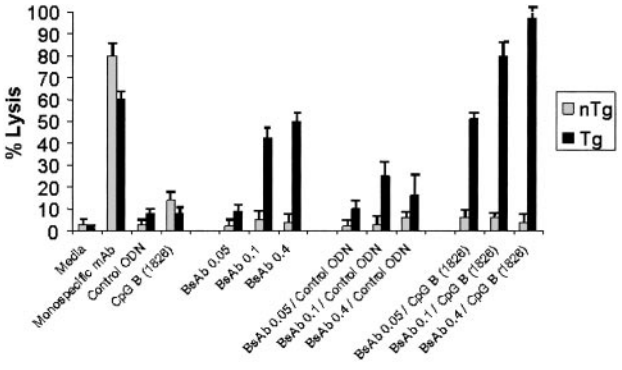

Fig. 5. CpG ODN B enhances ADCC mediated by anti-hFcγRI x anti-HER-2/*neu* BsAb. Tg and NTg mice were treated with mG-CSF for 3 days to increase granulocytes numbers. Whole blood was harvested from mice and incubated with various concentrations of monospecific anti-HER-2/*neu* mAb (*520C9*) or BsAb (*MDX-H210*), along with 10 μg/ml of CpG B or control ODN. Effector cells were mixed with ^{51}Cr-labeled SK-Br-3 cells as outlined in "Materials and Methods," and percentage specific lysis determined. All determinations were performed in triplicate in each experiment. Results from three independent experiments; *bars,* ±SD.

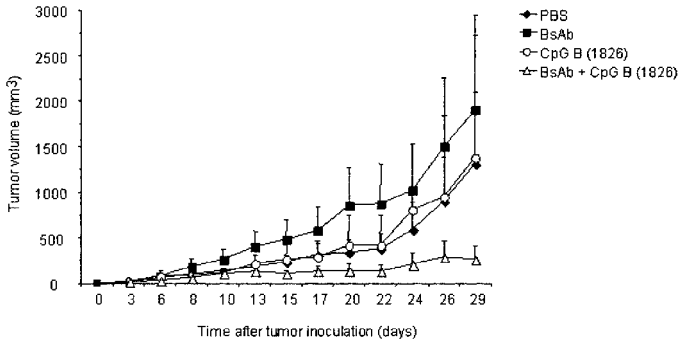

Fig. 6. CpG ODN B enhances the efficacy of anti-hFcγRI x anti-HER-2/neu BsAb therapy *in vivo*. hFcγRI-Tg were treated s.c. with 7.5 μg CpG ODN 1826 1 day before inoculation with CMS7HE tumor cells as described in "Materials and Methods." Mice were treated i.p. twice daily (on days 1–5 and 9–13) with 10 μg/100 μl of MDX-H210 or 100 μl PBS. On day 7 a second dose of 7.5 μg CpG ODN 1826 was administered. Tumor volumes, reported as length × width × height (mm^3), were measured three times per week, and toxicity evaluated. Results represent from three independent experiments; *bars*, ±SD.

cells played the central role, with the enhanced antitumor effect being lost after depletion of NK cells. In contrast, the antitumor effect of mAb plus CpG B could be mediated by either granulocytes or NK cells, because depletion of either population alone had little impact on efficacy, whereas depletion of both types of effector cells resulted in a loss of the therapeutic effect.

Use of Ab to deplete various cell populations has its limitations. Although we administered anti-NK and antigranulocyte mAb frequently with the goal of complete depletion, and were unable to find NK cells or granulocytes after depletion during the critical therapeutic window of the antitumor mAb, depletion may not have been complete. Residual NK cells or granulocytes may have contributed to the residual antitumor activity either by mediating ADCC or supplying cytokines that contributed to activating other cellular effectors. It is also possible that this approach resulted in unintentional depletion of a cell population that was important for the antitumor activity of therapy. This is particularly important in interpretation of the granulocyte depletion studies, which were done using the anti-LY-6C mAb Gr-1. Whereas this mAb clearly can deplete granulocytes, it has also been reported that the LY-6C target antigen is expressed by other cell populations, including plasmacytoid DC (42). The ability of this population of DC to produce IFN-α is enhanced by CpG ODN (43, 44). Thus, an alternative explanation for the observed findings is that depletion with Gr-1 decreased the efficacy of therapy by eliminating the IFN-α-producing cell. If this was the case, we would have expected depletion with Gr-1 to significantly decrease the therapeutic efficacy of mAb and CpG A, because the primary mechanism of action proposed for CpG A is induction of IFN-α production by plasmacytic DC, with secondary activation of NK cells. In fact, Gr-1 depletion had no detectable effect on therapy with mAb plus CpG A (Table 1), suggesting that our proposed mechanism, namely granulocyte depletion, and not depletion of IFN-α-producing cells, was responsible for the observed effects.

A second approach to exploring the role of various effector cells is to evaluate antitumor effects using BsAb that engage effector cells in a more focused manner. Granulocytes can express FcR and so could also contribute to the observed antitumor effects of mAb. *In vivo* studies in mice and data from clinical trials of the hFcγRI-directed BsAb, MDX-H210, have shown that granulocytes can be retargeted and demonstrate biological activity (8, 12, 45–49). To assess whether CpG ODNs enhance the efficacy of ADCC at least in part by inducing up-regulation of hFcγRI on granulocytes, we evaluated the effect of CpG B on hFcγRI expression and function. hFcγRI expression on murine granulocytes was up-regulated by a single low dose of CpG B *in vivo*. In addition, whole blood obtained from mG-CSF-treated hFcγRI Tg mice and stimulated *in vitro* with CpG B enhanced the ability of that blood to mediate ADCC. Up-regulation of hFcγRI on monocytes, macrophages, and DCs, and ADCC mediated by these cells, could have been responsible for some of the enhanced hFcγRI-mediated ADCC seen with CpG B. However, mG-CSF stimulated blood has low numbers of these cells. The E:T ratio would have been inadequate to mediate ADCC if the granulocytes were not contributing to the cytotoxic effect.

TLR9 is a key receptor in the response to CpG ODNs (50). A coreceptor responsible for the differential effects of CpG A and CpG B has been hypothesized but has not yet been identified. Production of a variety of cytokines by subsets of DCs (44) appears to be particularly important in the systemic response to CpG ODNs in both the murine and human systems. Interestingly, purified murine monocytes express TLR9, whereas purified human monocytes do not. This and other species differences related to response to CpG ODNs need to be taken into account when applying results in murine models to humans. Nevertheless, there are key similarities in the response of mice and humans to CpG ODNs. For example, in a recently completed Phase I clinical study, CpG ODN was found to enhance production of tumor necrosis factor α, interleukin 12, IFN-α, and IFN-γ. Some of the cytokines produced in response to CpG ODN in the human system, such as IFN-γ, up-regulate FcγRI and activate monocytes indirectly. Indirect mechanisms are also responsible for the effects that CpG ODN has on granulocytes, because TLR9 is not expressed by murine or human granulocytes. CpG A and CpG B have similar, although not identical, effects on immune cell populations and cytokine production in mice and humans. Thus, there is reason to hypothesize that the differences in response to CpG A and CpG B observed in the murine models described here could be found in humans as well.

In conclusion, the studies outlined above supply valuable information related to the cellular effector mechanisms responsible for the antitumor effects seen with mAb alone, and when combined with CpG A or CpG B. CpG A enhances the efficacy of mAb therapy largely by activating NK cells, whereas CpG B activates multiple effector cell populations. Both classes of ODN are effective only when used with mAbs that are potent mediators of ADCC. Granulocytes appear to play an important role, and may be responsible for much of the synergistic effect seen with the combination of mAb and CpG B. Human FcγRI expression on granulocytes is up-regulated by CpG B. This FcR is likely responsible for much of the enhanced lysis seen with BsAb and CpG B. A number of important questions remain. We do not know the contribution of monocytes and macrophages, because hFcγRI is also up-regulated on these cells by CpG B. It remains unclear whether the contribution of NK cells and granulocytes is because of their ability to mediate ADCC directly, or whether their role is more indirect because of production of cytokines that contribute to activation of other cell populations. Whereas animal models are useful, they do not always reflect the mechanisms responsible for the efficacy of mAbs used for human disease. Clinical trials of mAb plus CpG B, with clinical correlative studies, have begun recently, and will supply important information related to whether the promising results in animal models can be duplicated in the clinic. Ongoing preclinical and clinical evaluation of mAb mechanisms of action, including assessment of the role of monocytes and macrophages, and immunotherapeutic approaches in involving mAb plus other immunologically active agents is also needed if we are to determine which cellular effectors are responsible for the antitumor effects of mAb and use this information to develop the next generation of rationally designed immunotherapeutic approaches.

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