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Immunology

Biological activity of human–mouse IgG1, IgG2, IgG3, and IgG4 chimeric monoclonal antibodies with antitumor specificity

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ABSTRACT **Chimeric antibodies were constructed in which the murine variable region of anti-colorectal cancer monoclonal antibody CO17-1A was joined with human γ1, γ2, γ3, and γ4 constant regions. Human–mouse chimeric proteins were compared with the parental murine IgG2a antibody CO17-1A for their ability to participate in tumor-cell destruction by human and murine effector cells in antibody-dependent cell-mediated cytotoxicity (ADCC) assays. All of the chimeric antibodies showed different degrees of ADCC with human lymphocytes, monocytes, and granulocytes and with murine macrophages. Monocytes and macrophages were able to utilize the chimeric IgG1 and, to a lesser degree, IgG4 and IgG3 antibodies to lyse tumor-cell targets in ADCC assays. The chimeric IgG1 and IgG4 antibodies were nearly as effective as the parental CO17-1A antibody in inhibiting tumor growth in nude mice. These data indicate that chimeric IgG1 antibody is superior in its antitumor activity.**

Monoclonal antibody (mAb) CO17-1A was developed in 1978 (1, 2) and, after characterization of its antitumor activities (3, 4) and of its selective binding to colon carcinoma cells (5, 6), was used in clinical trials by Sears *et al.* (7, 8). CO17-1A is of the IgG2a isotype and is active in antibody-dependent cell-mediated cytotoxicity (ADCC) assays with murine (3, 9, 10) and human (9–13) effector cells. This mAb also inhibits the growth of human tumor xenografts in nude mice (3). In patients with colon and pancreatic cancer treated with mAb CO17-1A, complete and partial remissions have been described (7, 8, 14–17). However, murine immunoglobulins administered to humans have a short half-life (12–18 hr) and induce an anti-mouse immunoglobulin response (7, 8, 18). For these reasons, recombinant DNA techniques have been used to construct chimeric mAbs in which the human constant (C) region of choice is linked to the murine variable (V) region (19–21). A chimeric CO17-1A protein has been constructed with the human $C_{\gamma 3}$ region (22), and its characteristics have been described (23, 24). Recently, we used the cloned murine V region of CO17-1A to construct chimeric molecules with human $C_{\gamma 1}$, $C_{\gamma 2}$, and $C_{\gamma 4}$ regions. The availability of the series of chimeric mAbs with the same binding specificity permits direct comparison of all four human IgG isotypes for their effects *in vitro* and *in vivo* on human tumor-cell targets. Such analyses may provide a rationale for the selection of the most promising chimeric mAbs for clinical application.

MATERIALS AND METHODS

Chimeric Proteins. Methods for construction and characterization of γ1, γ2, γ3, and γ4 chimeric antibodies have been detailed (24). In brief, the functionally rearranged heavy- and light-chain V genes of hybridoma 17-1A were isolated and inserted into expression vectors containing genomic DNA segments encoding human γ heavy-chain and κ light-chain C regions. As described previously (24), transfection of these expression vectors containing mouse–human chimeric immunoglobulin genes into nonproducing mouse myeloma Sp2/0 cells resulted in stable cell lines producing IgG3(κ) chimeric antibody. The V gene of 17-1A was also linked to the human γ1, γ2, and γ4 C regions. The resulting chimeric heavy-chain genes were cotransfected with the chimeric light-chain genes into Sp2/0 cells to generate stable cell lines secreting IgG1(κ), IgG2(κ), and IgG4(κ) antibodies, respectively. The transfected cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 15% (vol/vol) fetal bovine serum (FBS) and containing antibiotic G418 at 1 mg/ml, mycophenolic acid at 1 μg/ml, xanthine at 50 μg/ml, and hypoxanthine at 2.5 μg/ml. The concentration of secreted antibody in the culture supernatant reached 10–15 μg/ml.

Purification of Chimeric IgG. Chimeric IgG1(κ), IgG2(κ), and IgG4(κ) antibodies were purified by passing the tissue culture supernatant over a protein A-Sepharose CL-4B (Pharmacia) column. Bound antibody was eluted in citrate buffer with a linear pH gradient of 3.5–5. Chimeric IgG3(κ) antibody was purified by sequential ion-exchange column chromatography on DEAE-Sepharose 6B and Mono S (Pharmacia) (23).

Cell Lines. U-937 is a myelomonocytic cell line derived from a patient with histiocytic lymphoma (25) maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, and 50 μg of gentamicin per ml. Colorectal carcinoma cell lines SW948 (colon) and SW707 (rectal) (3) and melanoma cell line WM164 (26, 27) have been described. These cell lines were maintained in Leibowitz' L15 medium (Hazelton Research Products, Denver, PA) containing 10% FBS, 2 mM glutamine, and 50 μg of gentamicin per ml.

Isolation of Effector Cells. Enriched preparations of human monocytes were obtained from normal donors by adherence selection of Ficoll-Paque-purified (28) peripheral blood leukocytes (PBLs) on gelatin/fibronectin-coated flasks (29). Natural killer/killer (NK/K) cells contaminating the monocyte preparations were lysed by incubation with anti-Leu-11b mAb and rabbit complement (29). Nonadherent leukocytes were obtained after three 1-hr adsorption periods on plastic surfaces. Human granulocytes were isolated from peripheral blood of healthy donors. The blood was mixed with 5% (wt/vol) Dextran T70 (Pharmacia, Uppsala, Sweden) in 0.9% NaCl (2.5 ml per 10 ml of blood) and incubated for 1 hr at 37°C. Leukocyte-rich plasma was then collected and centrifuged at 650 × *g* for 15 min at room temperature on discon-

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Abbreviations: mAb, monoclonal antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; PBL, peripheral blood leukocyte; NK cell, natural killer cell; FcR, receptor for Fc part of immunoglobulin; C, constant; V, variable; FITC, fluorescein isothiocyanate; E/T ratio, effector-to-target cell ratio; IFN-γ, interferon γ.

tinuous density gradients consisting of Ficoll-Paque (2.0 ml, upper layer) and Histopaque 1119 (Sigma) (3 ml, lower layer) in 15-ml tubes. Cells were harvested from the interface between Ficoll-Paque and Histopaque 1119 and resuspended in RPMI-1640 with 10% FBS (pH 7.4) after three washings. This suspension contained >98% granulocytes as judged by morphology of cells on stained slides. Thioglycolate-elicited murine peritoneal macrophages from 6- to 10-week-old CBA mice were prepared as described (5).

Flow Cytofluorometry. To detect binding of chimeric mAb to the receptor for the Fc portion of immunoglobulin (FcR), U-937 cells were treated with interferon γ (IFN-γ) (Interferon Sciences, New Brunswick, NJ) for 30 hr (30) and then incubated with chimeric mAb followed by fluorescein isothiocyanate (FITC)-conjugated goat antiserum to mouse $F(ab')_2$ fragment or rabbit antiserum to human IgG or Fc. Fluorescence was detected in an Ortho Cytofluorograf connected to an Ortho 2150 data-handling system (Ortho Instruments).

Cytotoxicity Assays. Subconfluent tumor-cell monolayers were labeled with [^{111}In]indium oxine (29) (indium oxyquinone, 20 mCi/ml; Medi-Physics, Emeryville, CA; 1 Ci = 37 GBq). The cells were suspended and added in triplicate to U-bottomed 96-well Linbro microtiter plates at 10^4 cells per well in RPMI-1640 containing 2% FBS (referred to as ADCC medium), after which 100 μl of mAb in the same medium was added. [^{3}H]Thymidine-release assays were carried out as described (31). Negative controls were murine anti-influenza mAbs (IgG2a) kindly provided by W. Gerhard (The Wistar Institute). Effector cells were added at the predefined effector-to-target (E/T) ratio in ADCC medium, and plates were incubated at 37°C in humidified 5% CO_2/95% air for 18 or 6 hr, as required. For ADCC assays, the percent specific cytotoxicity was calculated from ^{111}In-release or [^{3}H]thymidine release of test samples and control samples, as follows: % specific release = $[(E - S)/(M - S)] \times 100$, where E = experimental release (cpm in supernatant from target cells incubated with effector cells and experimental anti-target cell protein), S = spontaneous release [cpm in supernatant from target cells incubated with effector cells and control mAb of irrelevant (influenza virus) specificity], and M = maximum release (cpm released from target cells lysed with 2% Na-DodSO$_4$).

Inhibition of Tumor Growth. BALB/c *nu/nu* mice were inoculated subcutaneously with 5×10^6 SW948 colon carcinoma cells. Immediately after tumor-cell inoculation, mice received intraperitoneal injections of 100 μg of chimeric mAb daily for 5 days. Control animals received IgG2a anti-influenza virus mAb H24B5 (negative control) or parental murine IgG2a mAb CO17-1A (positive control). Tumor volumes were recorded weekly, as described (3).

RESULTS

Binding of Chimeric mAbs to FcR. Control murine mAb CO17-1A bound to the high-affinity FcR of γ-interferon-treated U-937 cells (30) and was detected only by FITC-conjugated goat anti-mouse $F(ab')_2$ (Fig. 1). Binding levels were similar among chimeric mAbs with human γ1, γ3, and γ4 C regions, and binding was detectable with FITC-conjugated anti-human and anti-mouse sera (Fig. 1). Little binding of chimeric IgG2 was detected.

ADCC Assay with Human Effector Cells. Freshly isolated, unfractionated human PBLs showed 15–35% specific lysis of SW707 rectal carcinoma target cells with the control murine IgG2a mAb CO17-1A, depending on E/T ratios (Fig. 2*A*). Chimeric IgG1 mAb mediated the highest lytic activity of PBLs against SW707 targets: 27.5% at an E/T ratio of 10:1, 43.6% at 20:1, and 36.5% at 50:1. Chimeric IgG2, IgG3, and IgG4 participated in SW707 cell lysis by human PBLs at

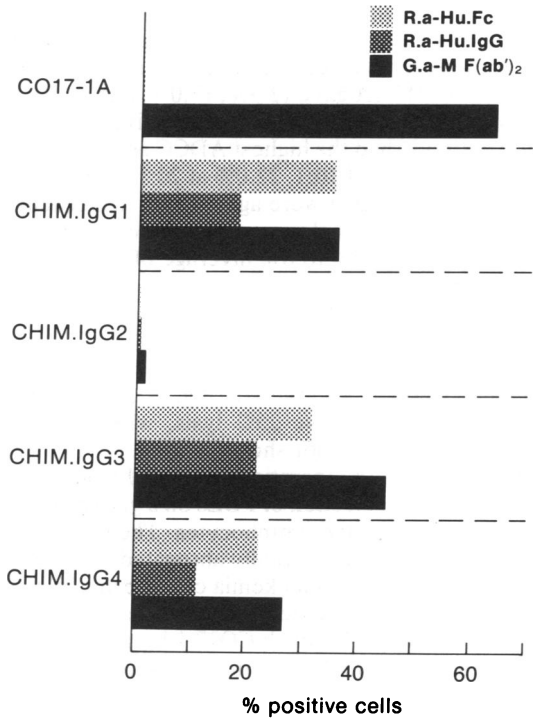

FIG. 1. Binding of chimeric (CHIM.) mAbs to FcR of U-937 cells treated for 30 hr with IFN-γ (500 units/ml). mAbs were used at 15 μg/ml. Flow cytofluorometry was performed to detect binding of mAbs with FITC-conjugated rabbit anti-human IgG Fc (R.a-Hu.Fc), rabbit anti-human IgG (R.a-Hu.IgG), or goat anti-mouse IgG $F(ab')_2$ [G.a-M $F(ab')_2$].

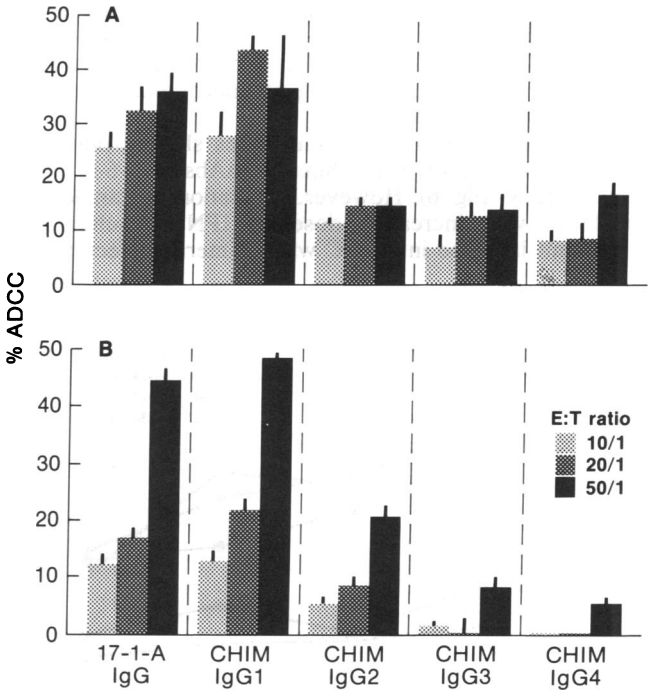

FIG. 2. ADCC against colorectal carcinoma cell lines with human PBLs as effector cells in 18-hr ^{111}In-release assay at E/T ratios of 10:1, 20:1, and 50:1. Results are presented as mean percent ^{111}In release (+SD) from triplicate samples of a representative experiment. SW707 rectal carcinoma (*A*) and SW948 colon carcinoma (*B*) cell lines were used as targets for lysis mediated by chimeric mAbs.

similar low levels, with an average of about 10% specific lysis at all three E/T ratios (Fig. 2*A*). In a similar experiment using SW948 colon carcinoma cells as targets (Fig. 2*B*), the control murine mAb CO17-1A gave 12.3%, 17.0%, and 44.5% lysis at E/T ratios of 10:1, 20:1, and 50:1, respectively. Again, IgG1 chimeric antibody had the highest ADCC activity with 13.0% lysis at an E/T ratio of 10:1, 22.0% at 20:1, and 48.3% at 50:1. IgG2, IgG3, and IgG4 were again much less reactive with PBLs against SW948 colon carcinoma cells. Spontaneous release ranged from 4% to 8% (average of 7%).

When human lymphocytes depleted of monocytes by adherence selection of PBLs were used as effector cells, two antibody groups could be distinguished, CO17-1A and chimeric IgG1 and IgG2, which kill well, and chimeric IgG3 and IgG4, which were not as effective (Fig. 3). At an E/T ratio of 50:1, IgG1 and IgG2 gave 25% lysis, where spontaneous release was 5.6% (data not shown).

Human monocyte preparations obtained from normal donors by adherence selection of PBLs on a gelatin/fibronectin surface contained 2–10% lymphocytes. The use of anti-Leu-11b mAb and complement totally abrogated NK-cell activity as tested on K562 erythroleukemia cells (results not shown). The NK-depleted monocyte preparation exhibited a significant level of ADCC with mAb CO17-1A, ranging from 9% at an E/T ratio of 10:1 to 29% at an E/T ratio of 100:1 (Fig. 4). Chimeric IgG1 had a similar ability to lyse target cells (13.5–25% lysis, depending on E/T ratio; Fig. 4). The other chimeras were lytic in the following relative order (according to release at the highest E/T ratio): IgG4 (5–25%), IgG2 (8–15%), and IgG3 (4–12%). Spontaneous release was 4–8.1%.

Human monocytes cultured for 2 days *in vitro* differentiate into macrophages and express higher levels of FcR (11, 12). Such 2-day-old monocytes were active in ADCC assays with control mAb CO17-1A (35.5%) and with chimeric IgG1 (52%) but were much less active with the IgG4, IgG2, and IgG3 chimeras (23%, 12%, and 10%, respectively). The presence of IFN-γ (500 units/ml) in the monocyte culture increased the level of lysis for mAb CO17-1A to 67.5%, for chimeric IgG1 to 78%, for chimeric IgG4 to 53%, for chimeric IgG2 to 36%, and for chimeric IgG3 to 35% (Fig. 5), under conditions where spontaneous release was 7.6%.

Freshly isolated human granulocytes showed only low-level ADCC activity with chimeric mAbs in a 6-hr ^{111}In-release assay (Fig. 6). However, incubation of granulocytes for 18 hr with increasing doses of IFN-γ resulted in a significant increase in ADCC with chimeric molecules, especially with chimeric IgG1 (9–25% with IFN-γ at 200 units/ml and 26% with IFN-γ at 1000 units/ml; Fig. 6).

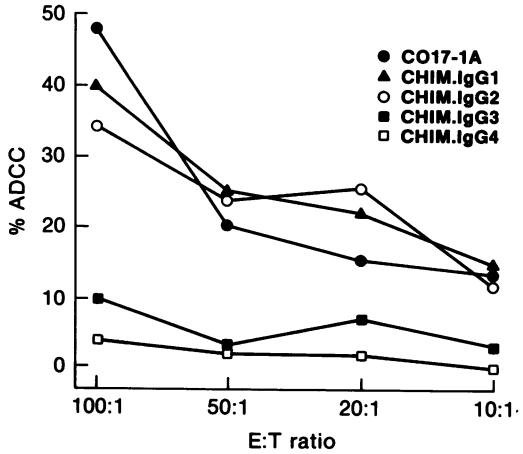

FIG. 3. Lysis of SW948 human colon carcinoma cells by chimeric mAbs and human lymphocytes (monocyte-depleted) in an 18-hr ^{111}In-release assay at various E/T ratios. Results are presented as mean percent ^{111}In-release from triplicate samples of one of three separate experiments.

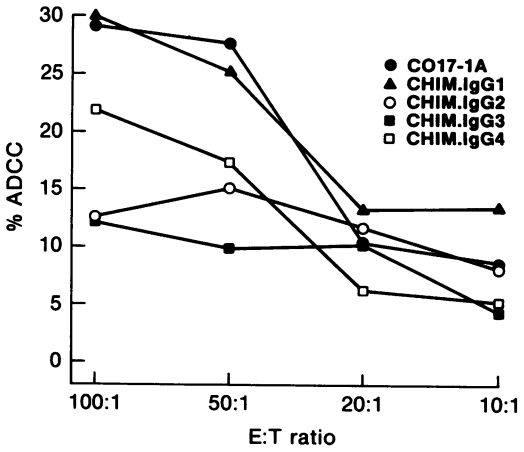

FIG. 4. Chimeric mAb-mediated ADCC of human monocytes (NK-depleted) against SW948 colon carcinoma cells in an 18-hr ^{111}In-release assay. The experiments were performed and evaluated as in Fig. 2.

Murine Macrophage ADCC. Thioglycolate-elicited macrophages are known to be highly efficient in killing target cells with mAb (5, 32). However, it has remained unclear whether human Fc can react with murine FcR. Therefore, we tested the chimeric proteins and murine macrophages for ADCC activity against human colon carcinoma cell line SW948 in a [^{3}H]thymidine-release assay. mAb CO17-1A and chimeric IgG1 lysed a similar percentage of cells, although CO17-1A was always slightly more active (Fig. 7). IgG4 induced about 50% lysis, whereas IgG3 was much less effective (25–30%) and IgG2 was totally inactive. The spontaneous release was always in the range 3.5–14% (average 12%). Similar results were obtained in three consecutive experiments.

Inhibition of Tumor Growth. mAb CO17-1A is known to inhibit growth of SW948 colon carcinoma cells xenografted into nude mice (5). Under the same conditions and antibody dosage, chimeric IgG1 and IgG4 were most efficient in inhibiting tumor growth, whereas chimeric IgG2 and IgG3 were ineffective (Fig. 8).

DISCUSSION

The chimeric mAbs described in this study contain the murine V region of mAb CO17-1A and human C domain γ1,

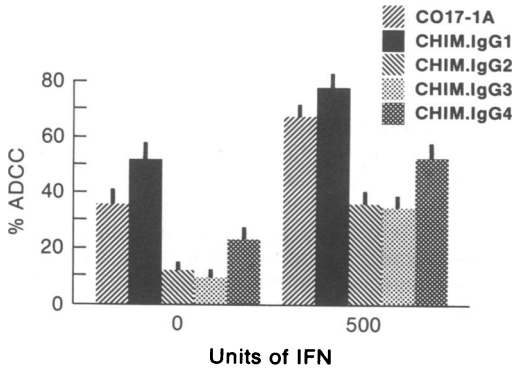

FIG. 5. Chimeric mAb-mediated ADCC of human monocytes (NK-depleted), cultured for 2 days and (where indicated) treated with IFN-γ at 500 units/ml for 30 hr, against SW948 colon carcinoma cells at a 50:1 E/T ratio. Results are presented as mean percent ^{111}In release (+SD) from triplicate samples of a representative experiment.

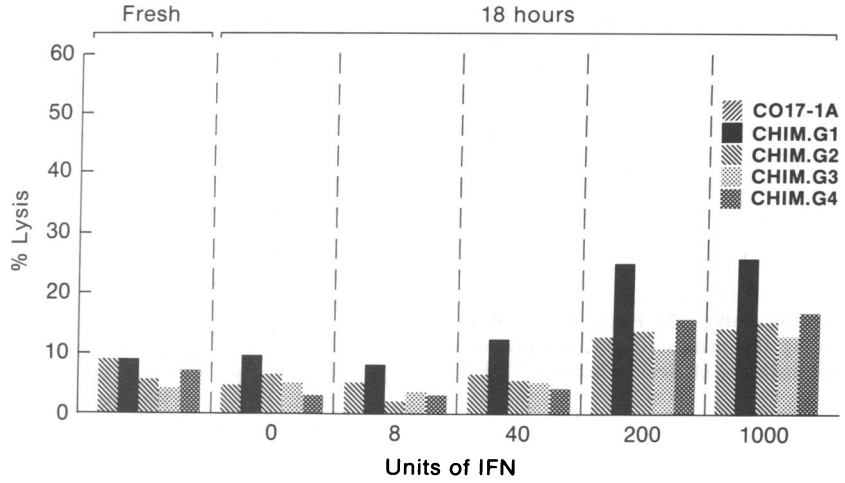

FIG. 6. Chimeric mAb-mediated ADCC of granulocytes against SW948 colon carcinoma cells in a 6-hr ^{111}In-release assay, at a 50:1 E/T ratio. Purified granulocytes were used as freshly isolated cells or after 18 hr of culture in the presence of IFN-γ as indicated (units/ml). Results are presented as mean percent ^{111}In release from triplicate samples.

γ2, γ3, or γ4 (19, 22, 24). The chimeric antibodies retained the binding specificity of mAb CO17-1A (24). Besides "transfectoma"-derived chimeric mAbs with hapten specificities (20, 21), similar chimeras have been produced that show antitumor activities (33–38). The data derived from these and similar experiments indicate that chimeric mAbs have biological functions (34, 38). However, not all human IgG heavy chains were examined. Here we describe the analysis of anti-colorectal carcinoma chimeric proteins representing all four human C regions. The chimeric antibodies showed significant ADCC activities with human and murine effector cells. Chimeric IgG1, representing human γ1 heavy chain, bound to the FcR of U-937 cells and participated in tumor-cell lysis in ADCC with all human effector cells, including total PBLs, monocytes, macrophages, lymphocytes (depleted of monocytes), and granulocytes, especially after IFN-γ induction (39). The chimeric IgG1 also participated in ADCC with murine macrophages, showing high cytolytic activity comparable to that described for the parental CO17-1A murine mAb (13). Chimeric antibodies with γ3 or γ4 heavy chains also bound to the FcR of U-937 cells and participated, although to a lesser degree, in ADCC with human PBLs, monocytes, macrophages, and granulocytes induced by IFN-γ to express FcR. The IgG4 chimeric antibody was also strongly active in ADCC with murine macrophages. However, it did not induce lysis of tumor cells by human lymphocytes. Finally, the IgG2 chimera showed minimal binding to the high-affinity FcR of U-937 cells, and although it had some ADCC activity with human PBLs and lymphocytes, it was only slightly lytic with human monocytes and inactive with murine macrophages, which are known to express a high-affinity FcR (29). U-937 cells express only FcR type I, and the ADCC found with IgG2 and lymphocytes as effector cells was due rather to participation of FcR type II.

From these data, it is clear that the cells with FcR types I, II, and III, such as human monocytes, macrophages, polymorphonuclear cells, and murine macrophages, are able to utilize chimeric IgG1 mAb and, to a lesser degree, chimeric IgG3 and IgG4 mAbs for lysis of human tumor-cell targets in ADCC. IFN-γ-induced expression of FcR on monocytes and granulocytes correlated with highest ADCC activity of chimeric IgG1. However, we do not know whether this increased killing is due only to higher FcR expression or whether IFN-γ also has the ability to increase killing by other mechanisms.

Since mouse macrophages were also able to efficiently kill human tumor cells with some of the chimeric proteins, especially the IgG1 and IgG4 chimeras, we tested all four chimeric mAbs for their ability to inhibit growth of human

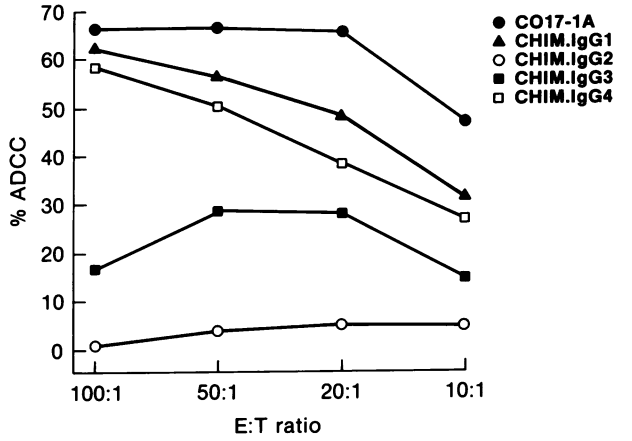

FIG. 7. Chimeric mAb-mediated ADCC of murine macrophages against human SW948 colon carcinoma cells in a 24-hr [^{3}H]thymidine-release assay, at various E/T ratios. Results are presented as mean percent [^{3}H]thymidine release from triplicate samples of a representative experiment.

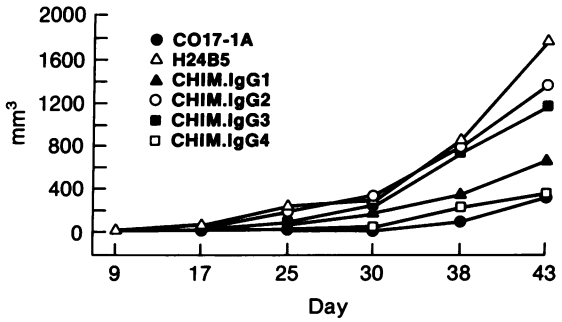

FIG. 8. Inhibition of tumor growth in nude mice by chimeric mAbs. Tumor cells were injected subcutaneously (5×10^6 SW948 cells per mouse), and mice (five per group) were treated for 5 days with 100-μg intraperitoneal injections of mAb. Positive control was the parental murine IgG2a mAb CO17-1A, and negative control was anti-influenza virus IgG2a mAb H24B5. Tumor size was monitored weekly by measuring three major diameters with graduated calipers, and the product of these three diameters was recorded as the tumor volume.

tumor xenografts in nude mice. The chimeric IgG1 and IgG4 mAbs were as effective as the parental CO17-1A in inhibiting tumor growth. IgG2 and IgG3 chimeras were not effective in the nude mouse system, as expected from their minimal participation in ADCC with murine macrophages, which are known effector cells for tumor destruction in this system (31).

The data indicate that IgG1 chimeric antibody is superior in its antitumor activity and may be considered for immunotherapeutic application.

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