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Murine Monoclonal IgG3 to Human Colorectal Tumor-associated Antigens: Enhancement of Antibody-dependent Cell-mediated Cytotoxicity by Interleukin 2[1]

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ABSTRACT

Murine monoclonal antibodies (MAB) of the IgG3 subclass generated to colorectal tumor-associated glycolipids were assessed for enhancement of antibody-dependent cell-mediated cytotoxicity (ADCC) by interleukin 2 (IL-2). Mononuclear cell preparations containing large granular lymphocyte effectors required only low doses of IL-2 (0.1–10 units/ml) and short exposure (3 h) for maximal enhancement of ADCC. Exposure of mononuclear cells for longer periods (1–6 days) to higher levels of IL-2 (100–1000 units/ml) resulted in the generation of lymphokine-activated killer N cells which were lytically active without antibody. A non-ADCC MAB of the IgG1 subclass showed no ADCC even after stimulation of effector cells with IL-2. Effector cells pretreated with IL-2 showed an enhanced rate of cytolysis of target cells in the presence of antibody. Pretreatment of effector cells with IgG3 MAB resulted in lower ADCC, but pretreatment of target cells or simultaneous addition of MAB and effectors to target cells gave higher levels.

These results indicated that ADCC with murine IgG3 is enhanced by levels of IL-2 achievable in current patient trials. The combination of antibody and IL-2-boosted effector cells gives comparable levels of killing to lymphokine-activated killer cells but should not suffer from similar toxicity. The NR-Co-04 antibody in combination with lymphokine may prove effective in treating colon cancer, a disease for which both chemotherapeutic and current biological therapies suggest a need for improved forms of therapies.

INTRODUCTION

In the preceding paper (1), we described the generation and characterization of murine IgG3 MAB[3] to colon tumor-associated antigens. These antibodies recognize glycolipids which are well expressed on colon tumors and some normal tissues. These antibodies are capable of mediating both ADCC with peripheral mononuclear cells as well as C′MC. The antibodies also demonstrated localization to nude mice xenografts of colon tumors with localization indices of greater than 10:1 at 8 days compared to an irrelevant IgG3 antibody.[4] The combination of tumor selectivity together with high effector capabilities suggested the use of these antibodies for treatment of colon cancer.

We have previously shown that murine IgG3 antibodies are the most effective subclass at mediating ADCC when compared to other murine IgG subclasses (2). However, not all IgG3 antibodies mediate ADCC and certain antibodies of other murine subclasses can also mediate ADCC. In addition, we have shown that this IgG3 subclass of antibodies interact predominantly with LGL, the activity of which can be augmented with either IL-2 or α-interferon. Additional evidence (3) obtained with class switch variants of a Thy-1.1 antibody has also demonstrated that IgG3 is the most effective subclass in mediating effector responses with LGL.

The potential clinical utility of such antibodies lies in the fact that they mediate both ADCC and C′MC. This is perhaps their most unique characteristic since antibodies like 17-1A directed against colon cancer are also effective in mediating ADCC by monocytes but do not fix human complement (4). Both functional capabilities may be necessary in the clinical situation to both interact with effector cells and attract them to the tumor site by generation of complement-derived chemotactic factors (5).

Previous results with other murine IgG3 to tumor-associated antigens of human melanoma and neuroblastoma have indicated that the antibodies alone can cause antitumor responses (6, 7). However, in both types of disease it was noted that the responses were only in a subset of the treated patients with no clear dose dependence. This may be related to individual patient variation in the ability to mediate ADCC since some patients have substantially suppressed ADCC (2, 8). Levels in patients who demonstrate suppressed ADCC can be increased *in vitro* to levels similar to those of normal donors by prior exposure of effector cells to IL-2 (8). If these results can be substantiated *in vivo*, the initial promising clinical results with murine IgG3 may be greatly improved.

In this paper we describe the enhancement of ADCC with murine IgG3 to colon cancer with mononuclear effector cells and IL-2. We have evaluated the effect of dose and time of exposure with IL-2 as well as the isotype dependence for enhancement. We have also demonstrated that protocols effective for LAK cell generation result in effector cells with no increase in lysis upon addition of antibody. Most importantly, we have shown that ADCC is not inhibited by human serum suggesting a high affinity binding between LGL Fc receptor and murine IgG3. These results indicate a potential utility for IL-2 enhancement of ADCC with murine IgG3 antibodies, specifically with the NR-Co-04 MAB in colon cancer.

MATERIALS AND METHODS

Antibodies. As demonstrated in the preceding paper (1), NR-Co-04 is the most effective of the murine IgG3 antibodies in mediating ADCC and C′MC of the series tested. Thus, this antibody was selected for use throughout these studies. NR-Co-04 was used as a purified immunoglobulin preparation. Antibody was produced by Damon Biotech (Boston, MA) and purified to 99% homogeneity by ion exchange chromatography. The antibody was stored in frozen aliquots at 2 mg/ml in a 0.1 M phosphate-buffered saline, pH 6.2. A second monoclonal with ADCC capability, NR-Lu-10, of the IgG2b subclass, was also used as well as an IgG1 anti-carcinoembryonic antigen antibody, NR-Co-02, which binds the same target cells. Human serum used in inhibition experiments was heat inactivated (56°C for 30 min) prior to use.

Cell Lines. The colon carcinoma cell lines LS-180, LS-174, and SW-1222 were all obtained from the ATCC cell culture collection and maintained in continual passage during these studies. Cells were grown

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[3] The abbreviations used are: MAB, monoclonal antibodies; ADCC, antibody-dependent cell-mediated cytotoxicity; C′MC, antibody-dependent complement-mediated cytotoxicity; IL-2, interleukin 2; LAK, lymphokine-activated killer cells; LGL, large granular lymphocytes; NK, natural killer.

[4] A. Charles Morgan, Jr., unpublished information.

in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum with no antibiotics. Cells at confluency were harvested by trypsinization and reseeded at high density into new flasks 1 day before use. Cells were then harvested on the day of the experiment with EDTA (0.001%) in phosphate-buffered saline and mechanical agitation.

Lymphokine. IL-2 was supplied by the Genetics Institute, Cambridge, MA, and was stored in aliquots in 0.1 M acetic acid at −70°C and diluted into medium before use. Preparations were standardized against a Biological Response Modifiers Program natural IL-2 preparation by comparing levels of IL-2 necessary for enhancing NK activity against LS-174 colon carcinoma cells. Endotoxin was assessed by a solid phase limulus amebocyte lysate assay supplied by Whitaker MA Bioproducts, Walkersville, MD, and found to be less than 1 pg/ml.

Isolation of Effector Cells and Incubations with IL-2. Peripheral blood was collected in heparinized tubes, mixed with an equal volume of lymphocyte separation medium (Organon Technica, Durham, NC). Tubes were centrifuged at 650 × *g* for 20 min at room temperature, and the mononuclear layer was collected free of polymorphonuclear contamination (less than 5%). Cells were then washed with phosphate-buffered saline and resuspended in assay medium (RPMI 1640) without glutamine (Whitaker MA Bioproducts) containing 5% heat-inactivated fetal calf serum. For short-term incubations with IL-2, effector cells were added to microtiter plates and incubated in medium with various concentrations of IL-2. For longer incubation, cells were incubated in 25-cm^2 flasks for up to 6 days with various concentrations of IL-2. Cells were collected by scraping if necessary and then added to test wells for ADCC.

ADCC. The assay was conducted described previously (2). Briefly, target colon carcinoma cells (usually LS-180) were harvested from exponentially growing cultures, rinsed, and diluted to 10×10^6 viable cells/ml. Target cells were then labeled with 400–500 μl of ^{51}Cr (1 mCi/ml; Du Pont-New England Nuclear, Boston, MA) for 2 h at 37°C with gentle mixing every 15 to 30 min. Labeled cells were washed twice in assay medium, and the final pellet was resuspended in assay medium and diluted to 2×10^5 cells/ml. In a typical assay, 50 μl of an appropriate concentration of IL-2 were added to wells except those to be used for determination of total and spontaneous release. Effector cells were then added in 100 μl of assay medium and then incubated at 37°C for 3 h. Antibody was added to test wells at 1 μg/well in 50 μl. Effector cells were added at ratios of 100, 50, 25, 10, and 1 to target cells. NK cell activity was assessed in wells with effector cells and an IgG1 antibody, NR-Co-02, which does not mediate ADCC. In all cases, incubation with non-ADCC antibody gave the same degree of lysis as effector cells only. Chromium-labeled target cells were added last and then the plate was centrifuged at 60 × *g* for 2 min and then incubated at 37°C for 3 h. Following the standard incubation or for other indicated times, the plate was again centrifuged and 100 μl of supernatant were removed and counted. All determinations were done in triplicate and the amount of ADCC was determined as

$$\%\ \text{of}\ ^{51}\text{Cr release} = \frac{\text{Experimental value} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100$$

Net lysis due to ADCC was calculated by subtracting the percentage of ^{51}Cr release due to effector cells with IgG1 non-ADCC antibody from these wells with effector cells with IgG3 antibody or wells with IL-2-activated effector cells with IgG3 antibody. Spontaneous release with the LS-180 cell line was generally less than 10% in a 4-h assay.

Enhancement of ADCC with IL-2. Each experiment for enhancement of ADCC with IL-2 was performed with at least three normal donors with results with a representative donor presented.

RESULTS

NR-Co-04 was tested for ADCC against different colon tumor cell lines (Table 1). The antibody was capable of lysis with mononuclear effector cells of all the colon tumor target cell lines tested; lysis of the LS-180 cell line was consistently high and it had a low NK background. Therefore, this cell line was selected for further evaluation of augmentation.

Table 1 *ADCC with MAB of different isotypes to carcinoma cell lines*

MAB	Subclass	% of ^{51}Cr release		
		LS-180	LS-174	SW-1222
NR-Co-01	IgG3	34.0[a]	48.5	20.2
NR-Co-03	IgG3	36.3	47.1	22.6
NR-Co-04	IgG3	39.0	60.7	31.9
NR-Co-05	IgG3	30.0	28.3	23.0
NR-LU-10	IgG2b	8.7	9.8	7.8
NR-Co-02	IgG1	3.6	7.7	4.4

[a] Lysis at effector:target ratio of 100:1 with 1 μg/ml of each antibody.

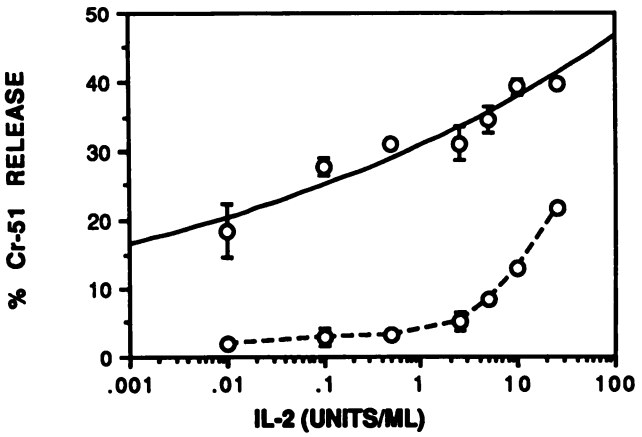

Fig. 1. Augmentation of ADCC with IL-2. Dose dependency. Mononuclear cells were incubated in microtiter plates with the indicated concentrations of IL-2 for 3 h. Radiolabeled target cells and antibody were then added. Effector:target ratio, 50:1. O——O, ADCC with NR-Co-04 MAB (IgG3) and IL-2-activated effector cells; O- - -O, activated effector cells only or NR-Co-02 MAB (IgG1) with activated effector cells.

Mononuclear cell preparations were exposed to IL-2 for 3 h at the indicated dose levels. Both ADCC and NK killing were assessed. As shown in Fig. 1, the concentration of IL-2 required for enhancement of ADCC was lower than that required for activating NK killing. Very low doses of IL-2 (0.1–1 unit/ml) provided for enhanced ADCC without appreciable boosting of NK killing. The low level of IL-2 required for enhancing ADCC contrasted to the higher doses of IL-2 (100–1000 units/ml) required for optimal LAK cell generation. In order to determine whether LAK cells could also mediate ADCC, we exposed mononuclear cell preparations to conditions optimal for LAK cell generation, *e.g.*, 1000 units/ml for 6 days. As shown in Fig. 2*B*, LAK cells were generated which were active against the colon tumor target cells. However, there was no increase in effector cell killing with added IgG3 antibody. The same donor exposed for only a brief interval, *e.g.*, 3 h, to 100 units/ml showed enhanced ADCC (Fig. 2*A*).

Having established the fact that short exposure of mononuclear cells to low concentrations of IL-2 was effective in enhancing ADCC with murine IgG3, we sought to characterize the enhanced lysis ADCC with regard to the kinetics of killing and the ability to be inhibited by γ-globulins in human serum. As shown in Fig. 3, when effector cells were tested for ADCC activity following short exposure to IL-2, more rapid kinetics of lysis was seen with the IL-2-activated effectors than with nonactivated effectors. However, the level of killing was dependent upon the order of addition of the reagents to the assay system (Fig. 4). The simple addition of antibody, effectors, and target cells simultaneously to the well followed by centrifugation gave the highest percentage of ^{51}Cr release compared to preincubation of antibody with either effectors or target cells. If preincubation was followed by washing, there was a further

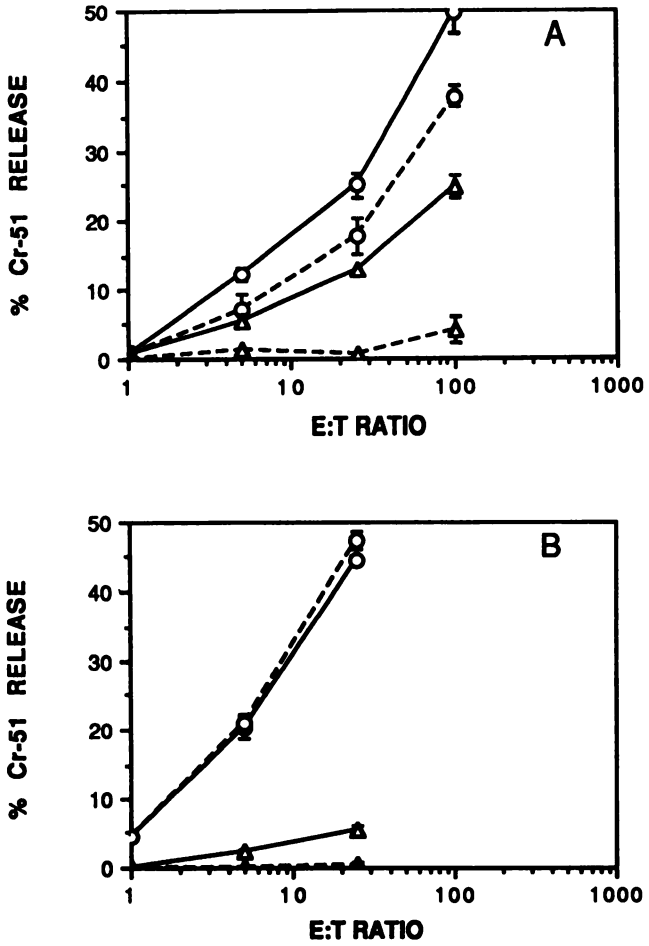

Fig. 2. Augmentation of ADCC with IL-2. Length of exposure of effector cells. Mononuclear effector cells were incubated for 3 h (*A*) to 6 days (*B*) with 1000 units/ml of IL-2. Shown are the shortest and longest exposure times for: O——O, NR-Co-04 + IL-2 activated effectors; O- - -O, NR-Co-02 + IL-2-activated effectors; Δ——Δ, NR-Co-04 + nontreated effectors; Δ- - -Δ, NR-Co-02 + nontreated effectors. Activated effector cells alone gave the same degree of lysis with or without NR-Co-02. Cultures exposed to 10 units of IL-2 maintained augmented ADCC throughout the 6-day culture period. Cultures incubated with 100 units/ml showed the same results as cultures with 1000 units/ml. *E:T*, effector:target.

diminution of cytolysis (not shown). These differences were seen with four different donors and indicate that it is important to continually expose target cells to antibody for maximum cell lysis.

Fc receptor binding of murine IgG2a MAB to human monocytes and potentially ADCC can be inhibited by human γ-globulin (10). We wished to determine whether γ-globulin in human serum would similarly affect ADCC mediated by IgG3 MAB with LGL effectors. As shown in Table 2, ADCC was not inhibited by concentrations of human serum up to 50%. These results indicate that the binding of the murine IgG3 to the Fc receptor on mononuclear effector cells (LGL) is of very high affinity and can likely occur in the *in vivo* situation.

DISCUSSION

We have demonstrated that the NR-Co series of murine IgG3 to colorectal tumor-associated antigens generated by solid phase lectin/extract immunizations were effective mediators of both ADCC and C'MC, similar to murine IgG3 generated to human melanoma and neuroblastoma (6, 7, 12). IL-2 is capable of augmenting ADCC at low doses and with short exposure. In contrast, LAK cells generated by prolonged exposure to high levels of IL-2 were optimally lytic and showed no enhancement upon addition of antibody. This is in contrast to results obtained with murine LAK cells which showed demonstrable ADCC activity (13). It is uncertain what the differences are between the two results. It has been found that murine LAK generated from spleen cells have a large T-cell component (14). Human LAK generated from peripheral blood have a large LGL component (15). From our own data and those of others, it appears that the LGL is the primary mononuclear effector cell for murine IgG3 ADCC and when exposed to high concentrations of IL-2 for long periods become optimally induced for non-antibody-targeted cytolysis but are perhaps down-regulated with

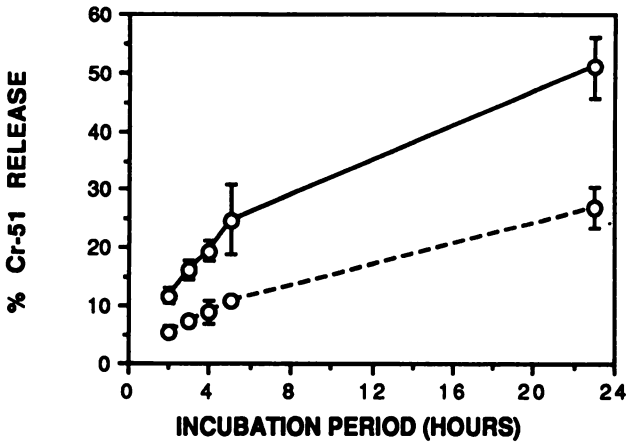

Fig. 3. Augmentation of ADCC with IL-2. Kinetics of cytolysis. Effector cells were exposed to 50 units of IL-2 for 3 h and then incubated on target cells with antibody for periods of 2 to 23 h. Background release at 23 h was 26%. Effector:target ratio was 25:1. O——O, NR-Co-04 with IL-2 activated effectors; O- - -O, NR-Co-04 with nontreated effectors.

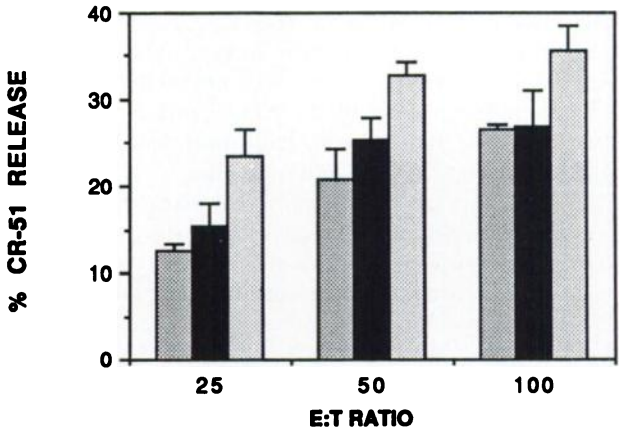

Fig. 4. ADCC: Effect of incubation conditions. For ⊞, effector cells were preincubated with antibody and then added to target cells; for ■, antibody was preincubated with target cells and then added to the effector cells; for □, effector cells, antibody, and target cells were added to wells simultaneously. ADCC was then performed with a standard 4-h ^{51}Cr release assay. No IL-2 was added. Effector:target (*E:T*) ratio, 100:1.

Table 2 *Lack of inhibition of ADCC with human serum*

% of human serum[a] (final)	% of ^{51}Cr release at effector:target ratio of		
	50:1	25:1	12:1
0	26.5	18.8	15.6
12	32.4	28.2	26.0
25	27.3	28.6	24.6
50	25.9	23.3	23.6

[a] Heat-inactivated human AB serum had no toxic effects on the target cells alone. Human serum was added to effectors in microtiter plates for 30 min at 37°C. Labeled targets and antibody (1 μg/well) were then added giving a final effector:target ratio of 100:1.

regard to Fc receptor expression and thus ADCC. Murine LAK may not be susceptible to this down-regulation. Alternatively, our results could also indicate that LAK cells may be optimally active against colon cancer target cells and, therefore, no additional killing can be seen by addition of antibody. To address this, either a cell line resistant to LAK killing should be used for ADCC testing or LAK killing should be blocked in order to see antibody-dependent killing. Regardless, our results are consistent with the concept that LGL treated with IL-2 represent a continuum of activation with low doses and short exposure giving rise to a highly lytic effector cell that is enhanced in its function by the addition of a bridge of antibody. At the other end of the continuum is an effector cell treated with high doses for long periods which are optimally lytic without antibody.

It also appears from current data that the short exposure of effectors to low concentrations of IL-2 gives equivalent lysis with murine IgG3 as non-antibody-mediated cytolysis with LAK cells. All of these findings would indicate that patients who are receiving IL-2 by a variety of clinical protocols would likely demonstrate an enhancement in ADCC in peripheral blood even without demonstration of substantial LAK activity. It has been the finding that with i.v. administration of IL-2, LAK activity is generated in peripheral circulation only after high dose levels which also causes Grade III and IV toxicity (16). Thus, it would be envisioned that for optimal combination with murine IgG3 MAB, only moderate, less toxic levels of IL-2 should be administered to patients. Because most protocols require sustained exposure to IL-2 to cause any degree of peripheral mononuclear cell activation, it is likely that it would take several days to weeks of IL-2 infusion to create a sustained activated state for ADCC.

Any *in vivo* ADCC applications of murine IgG3 MAB are obviously dependent upon the ability of the effector cell to interact with the antibody at the target site in the presence of high concentrations of irrelevant human IgG. Indeed, we showed that ADCC with murine IgG3 is not inhibited by human serum. We could, however, inhibit ADCC with very high doses of irrelevant IgG3 (not shown), indicating that even without antigen binding IgG3 binds with high affinity to Fc receptors on LGL. LGL have been shown previously to have Fc receptors that recognize aggregated immunoglobulin with a higher affinity than monomeric (19). The same Fc receptors would presumably bind antigen-complexed antibody with higher affinity than monomeric.

Thus, we have evaluated the ability of IgG3 MAB to interact with activated effector cells treated with IL-2. These results indicate that combination trials with the use of IL-2 with murine IgG3 against colon cancer are feasible. In addition, we are currently exploring combinations of lymphokines which also may be effective in boosting ADCC. Preliminary data indicate that murine IgG3 is also effective with activated monocytes.[5] Other results indicate that murine IgG3 also mediates ADCC with activated polymorphonuclear leukocytes.[6] Thus, the ability of murine IgG3 to mediate ADCC with multiple effector populations may be utilized to enhance treatment of solid tumors.

[5] R. A. Mufson and J. Aghajanian. Recombinant human macrophage colony stimulating factor (r-huM-CSF) specifically enhances macrophage tumoricidal activity in antibody dependent cell-mediated cytotoxicity (ADCC), submitted for publication.

[6] S. Graves, W. Sullivan, and A. C. Morgan, Jr. Murine monoclonal IgG3 antibodies to human colorectal associated antigens: activation of polymorphonuclear leukocytes (PMN) for ADCC with recombinant human granulocyte-monocyte colony stimulating factor (r-huGM-CSF), submitted for publication.

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