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Biological activity in the human system of isotype variants of oligosaccharide-Y-specific murine monoclonal antibodies*

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Summary. The capacity of isotype variants of BR55-2, an anti-tumor monoclonal antibody directed against Y oligosaccharide, to mediate antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) in the human system was evaluated using freshly isolated peripheral blood mononuclear cells, lymphocytes, monocytes and complement. The ADCC activities of the BR55-2 IgG3 isotype and its switch variants (IgG1, IgG2b, and IgG2a) with human monocytes were high for all isotypes, whereas the activity of all isotypes was lower with freshly isolated lymphocytes, IgG1 being the least effective. The CDC on the other hand was strong with IgG3, IgG2b and IgG2a and negative with the IgG1 variant. The IgG3 and IgG2a isotypes were selected for further development. Their strong ADCC and CDC activity against mammary carcinoma, colon carcinoma and small-cell lung tumor cell lines was confirmed quantitatively using proteins highly purified from tissue-culture supernatants. Significant variations in ADCC and CDC competence was observed among human donors.

Key words: Y oligosaccharide antigen – mAb isotype variants – Human effector mechanisms

Introduction

A considerable body of data has been accumulated suggesting the usefulness of murine anti-tumor monoclonal antibodies for the immunotherapy of human cancer. Several immunotherapeutic trials of human cancer using mAbs showed encouraging results [3, 5, 6, 9, 16–18]. The mechanisms involved in such antibody-directed tumor remission remain obscure. The effects may result from a combination of several different potential effector mechanisms, including mAb-mediated cytotoxicity with monocytes, macrophages, polymorphonuclear leukocytes, lymphocytes, natural killer cells (NK/K) and complement-dependent cytotoxicity. mAbs are known to participate in ADCC and CDC against target cells depending on their isotype [7, 14]. Effectiveness of a monoclonal antibody in these in vitro assays is an essential requirement for further development of mAbs for immunotherapeutic clinical trials. In this study ADCC and CDC activities of the IgG3 isotype of BR55-2 murine antitumor monoclonal antibody and its isotype switch variants [20, 21] were studied with human effector cells and human complement.

Materials and methods

Monoclonal antibodies. The production, isolation and characterization of BR55-2 IgG3 and its isotype switch variants have been described [20, 21]. mAbs for ADCC and CDC experiments were obtained from ascitic fluid produced in pristane-primed mice. Highly purified mAbs were obtained from tissue-culture supernatant for ^{51}Cr assays using conventional anion and cation chromatography. Purified human immunoglobulin G was purchased from Sigma, St. Louis, Mo., resuspended in RPMI media and ultracentrifuged. No aggregates were present in the final preparation.

Tumor cell lines. The human tumor cell lines used in this study including SW707 (rectal carcinoma), SW948 (colon carcinoma), SKBR5, and MCF7 (mammary carcinoma), and H-69 (small-cell lung carcinoma) have been described [1, 8, 11, 19].

Isolation of human effector cells. Peripheral blood mononuclear cells were obtained from healthy donors by Ficoll/Hypaque gradient centrifugation [2]. Enriched monocyte preparations were prepared from these cells by adherence selection on gelatin/fibronectin-coated flasks [4]. Contaminating NK/K cells were lysed by incubation with Leu11b mAb and rabbit complement. Non-adherent lymphocytes were obtained following three 1-h adherence periods on plastic surfaces. Serum from healthy donors was used as a human complement source.

Cytolytic assays. ADCC in ^{111}In-release assays was performed as described before [14]. Briefly, subconfluent tumor cell monolayers were collected and labeled with [^{111}In]indium oxine [10, 23] (indium oxy-

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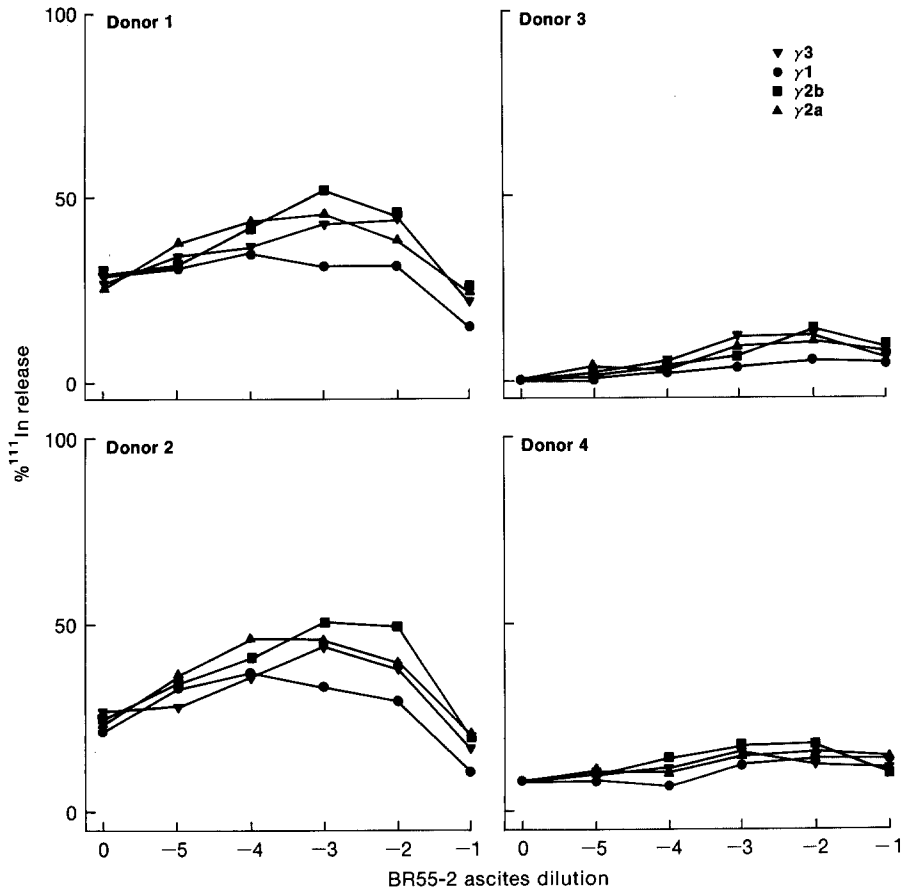

Fig. 1. Human-lymphocyte-mediated antibody-dependent cellular cytotoxicity (ADCC) of mAb BR55-2 IgG3 and its isotype switch variants against ^{111}In-labeled SW707 human rectal carcinoma cells. E : T ratio = 20 : 1. Four different donors were tested

quinone, 2.0 mCi/ml; Medi-Physics, Emeryville, Calif. and added in triplicate to U-bottomed 96-well Linbro microtiter plates at 1×10^4 cells/well in RPMI-1640 medium containing 2% fetal bovine serum, after which 100 µl ascites of mAbs diluted to the desired concentration was added. Effector cells were added at a predefined E : T ratio in RPMI-1640 medium/2% fetal bovine serum and the plates were incubated at 37° C in a humidified atmosphere of 5% CO_2 for 18 h.

ADCC in ^{51}Cr-release assay. The tumor cells were labeled with Na_2 $^{51}CrO_4$ (Amersham, Arlington Heights, Ill.) and 2.5×10^4 cells/well were added in triplicate to flat-bottomed 96-well microtiter plates containing the human peripheral blood mononuclear cells as effector cells at a predefined E : T ratio. Afterwards, 40 µl mAb solution, diluted to the desired concentration in phosphate-buffered saline (PBS), was added and the plates were incubated for 4 h at 37° C/5% CO_2.

CDC in ^{111}In release. The tumor cells were labeled as above, and 100 µl/well, containing 2.5×10^4 cells, was added in quadruplicate to flat-bottomed 96-well microtiter plates. The 40-µl mAb solution, diluted to the desired concentration, was added and the cells were incubated at 37° C/5% CO_2. Afterwards, 100 µl human serum/well was added as complement source (healthy donors) and the cells were incubated for 1 h at 37° C and 5% CO_2.

CDC in ^{51}Cr-release assay. The tumor cells were labeled as above and 2.5×10^4 cells/well were added in quadruplicate to flat-bottomed 96-well microtiter plates. Samples of 40 µl mAb solution, diluted to the desired concentration in PBS, was added and the cells were incubated for 2 h at 37° C/5% CO_2. Afterwards, 100 µl human serum/well was added as complement source (healthy donors) and the cells were incubated for 1 h at 37° C and 5% CO_2.

In all assays, the supernatants were harvested and counted in a gammacounter.

The percentage specific cytotoxicity for all assays was calculated from radioactivity released by the test sample and control samples, the spontaneous release and the maximal release as follows:

$$\text{Specific lysis (\%)} = \frac{E-S}{M-S} \times 100$$

where E = radioactivity (cpm) in supernatants from target cells incubated with effector cells or complement and experimental anti-(target cells) mAb, S = spontaneous release (cpm) in supernatants from target cells with (^{111}In-release assay) or without (^{51}Cr-release assay) mAb of irrelevant specificity and M = maximum release (cpm) from target cells, determined after NP40 lysis.

Statistical considerations. In all assays, each point represents the mean value of the radioactivities of three wells. Variations of radioactivity between wells were always less than 1%. Data from representative single experiments are presented since reproducible results were obtained in more than three experiments for each of the assays.

Results

The BR55-2 IgG3 isotype and its three switch variants were tested in the first set of experiments against the ^{111}In-labeled SW707 cell line in ADCC with human lymphocytes, monocytes and in CDC with human serum as a source of complement.

Human lymphocytes as effector cells of two donors were effective in ADCC and gave 30%–50% lysis depending on mAb isotype, the γ1 variant being the least effective (Fig. 1). Lymphocytes of donors 3 and 4 were totally ineffective in mediating ADCC with all four isotypes (Fig. 1).

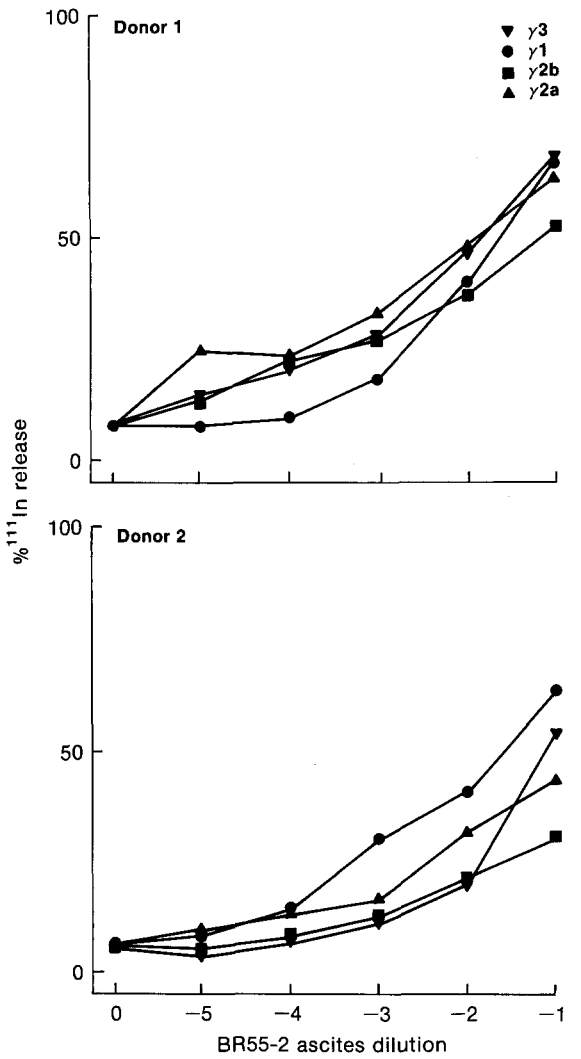

Fig. 2. Human-monocyte-mediated ADCC of mAb BR55-2 IgG3 and its isotype switch variants against ^{111}In-labeled SW707 rectal carcinoma cells. E : T ratio = 20 : 1

On the other hand, using a human monocyte preparation in the ^{111}In-release assay, the ADCC activities of all isotypes were found to be efficient, the IgG2b variant being slightly less active (Fig. 2). In these assays, there was also clear evidence of donor variability, with some donors showing as little as 20% lysis (results not shown).

After treatment of the adherence-selected monocyte preparation with mAb Leu11b and complement, which effectively abrogates all NK activity [3], significant levels of ADCC activity were still achieved comparable to levels seen in non-depleted effector cell populations (Table 1).

In order to confirm the participation of Fc receptor in ADCC, effector cells were exposed to purified non-aggregated human IgG and then used in an ADCC assay with the constant presence of human protein (Table 2)

As shown in Table 2, ADCC of murine BR55-2 IgG3 antibody could be inhibited from 68.6% to 12.4% in donor 1 and from 65% to 10.2% in donor 2, by preincubation with 2.4 mg/ml normal human IgG protein.

When human plasma was used as a source of complement in the ^{111}In-release assay, the high CDC activity was

Table 1. Antibody-dependent cellular cytotoxicity (ADCC) activity of mAb BR55-2 (IgG3) with natural-killer-depleted monocyte preparations (^{111}In release; SW707; E : T = 50 : 1)

Donor	BR55-2, IgG3	ADCC (%) with	
		Monocytes	Leu11b-treated monocytes
1	–	13	14
	+	69	72
2	–	14	11
	+	65	60

Table 2. Purified human IgG Inhibition of ADCC

Donor	BR55-2, IgG3 (1 : 50)	Lysis (%) in ADCC against SW707 cells	
		RPMI (10% FBS)[a]	Human IgG (2.4 mg/ml in RPMI (10% FBS)
1	–	13.3	6.6
	+	68.6	12.4
2	–	13.9	6.5
	+	65.0	10.2

[a] FBS, fetal bovine serum

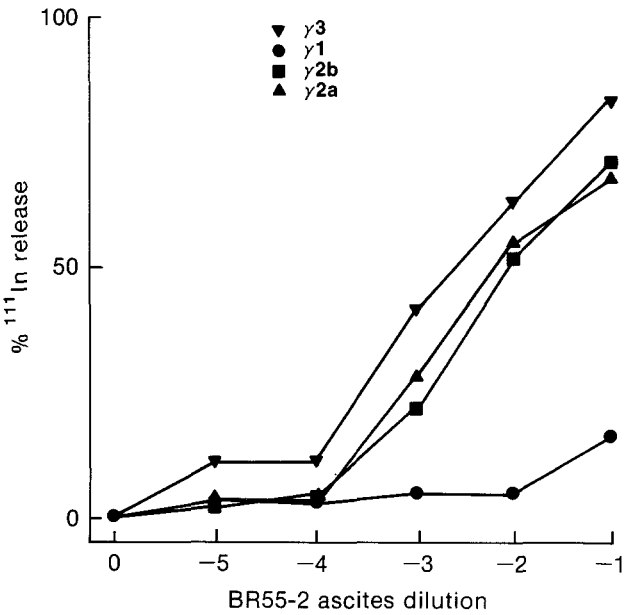

Fig. 3. Rectal carcinoma SW707 cell lysis in complement-dependent cytotoxcity by mAb BR55-2 IgG3 and its isotype switch variants. Target cells were labeled with ^{111}In. Each point represents mean value of triplicate wells

detected with IgG3, IgG2a, and IgG2b isotypes and negative results were obtained with the IgG1 variant, against human rectal carcinoma cell line SW707 (Fig. 3).

On the basis of these results, two isotypes (IgG2a and IgG3) were selected for further testing as a purified protein preparation, to exclude a possibility of interference by other proteins present in ascitic fluid. Both isotypes were purified from tissue-culture supernatants by conventional cation and anion chromatography.

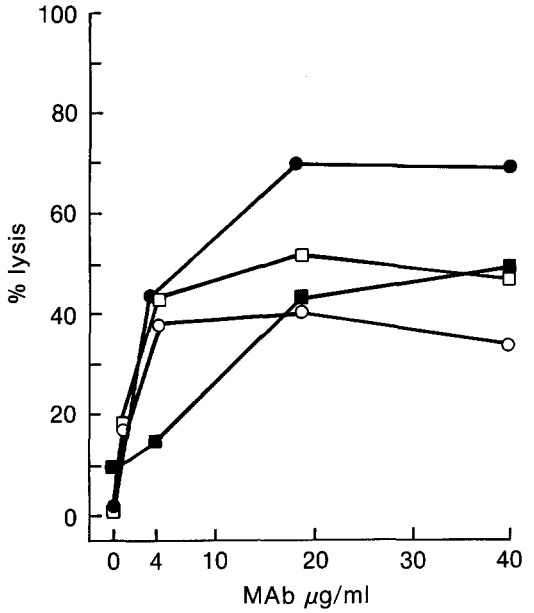

Fig. 4. ADCC activity of BR55-2, IgG2a and IgG3 isotypes. ^{51}Cr-labeled tumor cells (SKBr-5) were incubated with different mAb concentrations and human peripheral blood mononuclear cells (as effectors) for 4 h. *Symbols* represent different mAb isotypes: ○, □, IgG3; ●, ■, IgG2a. E:T = 20:1 (IgG3), E:T = 35:1 (IgG2a), on two different donors

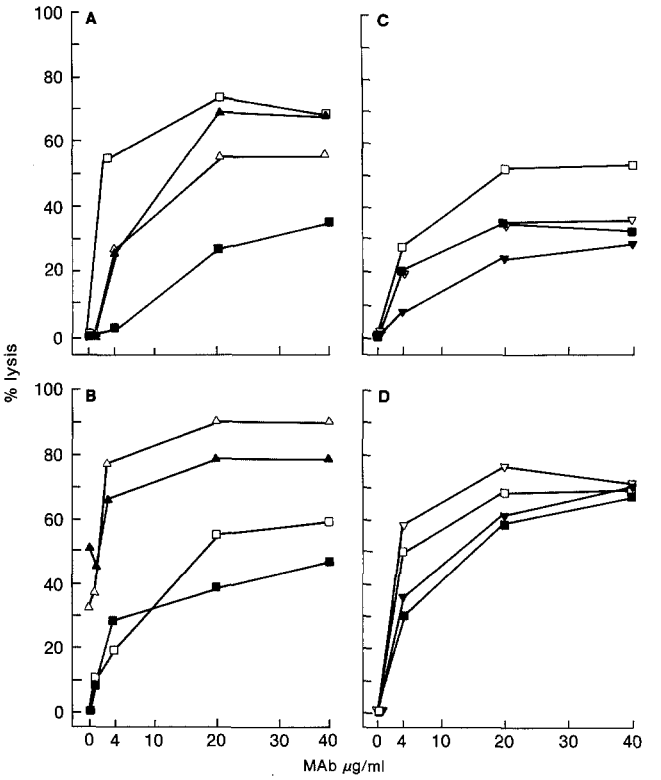

Fig. 5. Complement-dependent cytotoxicity activity of BR55-2, isotypes IgG2a and IgG3 against ^{51}Cr-labeled tumor cell lines. SKBr5 (4A) and H-69 (4B), MCF-7 (4C) and SW948 (4D) cells were preincubated with different concentration of mAbs for 2 h. Human serum was used as complement source, incubation time 1 h. *Symbols* represent different antibody isotypes: □, △, ▽, IgG3; ■, ▲, ▼, IgG2a, in two different donors (▲, △, ▼, ▽, donor 1; □, ■, donor 2)

In the 4-h ^{51}Cr-release assay with the total population of human peripheral blood mononuclear cells as effectors, both isotypes were effective in ADCC lysis of the breast carcinoma cell line SKBr5 (Fig. 4). Both donors showed the ability to lyse tumor cells in the range of 40%–70% (Fig. 4). Both highly purified isotypes, IgG3 and IgG2a, mediated tumor cell lysis, showing the expected donor and concentration relationships.

All four cell lines tested for CDC, breast carcinoma SKBr5 (Fig. 5A), small-cell lung carcinoma H-69 (Fig. 5B), breast carcinoma MCF7 (Fig. 5C) and colon carcinoma SW948 (Fig. 5D) were susceptible to the lysis by human complement. In all cases, IgG3 had slightly higher CDC activity, although the IgG2a variant was also clearly effective. Both breast carcinoma cell lines were efficiently lysed with clear donor-to-donor variation. Similarly susceptible to complement lysis was the colon carcinoma cell line SW948 (65%–75%). The small-cell lung carcinoma line H69 was also susceptible, although the percentage of lysis was lower (30%–50%).

As shown in Fig. 5, the CDC activity (^{51}Cr-release) of these two highly purified isotypes against four different tumor cell lines was highly efficient, the IgG3 isotype being more effective. The ^{111}In-release assay (Fig. 3) gave similar results for CDC reactivities of the IgG3 and IgG2a isotypes.

Discussion

Antibody-mediated tumor destruction is thought to follow two general mechanisms, antibody-dependent, cell-mediated cytolysis (ADCC) and complement-dependent cytolysis (CDC). In the present study the IgG3, IgG2a, IgG2b isotypes of BR55-2, an anti-tumor mAb, showed high activity in the in vitro CDC, whereas the IgG1 variant was inactive. All four isotypes exhibited in vitro monocyte-mediated ADCC activity, the IgG2b being slightly less active. We have used two assay systems, in which target cells were labeled with either ^{111}In or ^{51}Cr, and both assays gave comparable results in ADCC and CDC. It is not yet clear, which of these IgG subclass proteins might cooperate optimally with human effector systems in vivo (i.e. in the presence of serum IgGs, at high concentrations that might compete for effector FcRs) [12]. From the murine system experiments, it is clear that the IgG2a and IgG3 subclass of BR55-2 exhibit superior tumor-suppressive activity in vivo [22], despite the presence of high levels of murine IgG in circulation. We have previously shown that all isotype switch variants have the same affinity as parental IgG3 for antigen, and expected affinities for the Fc receptor [14, 21]. This permitted us to compare biological functions of these four isotypes directly. We have clearly shown that IgG3 and IgG2a isotypes can effectively utilize human effector mechanisms, both cellular and complement-dependent, for tumor cell destruction.

Our observations, together with the finding that generally murine IgG2a and IgG3 compete favorably with human IgG1 for binding to the high-affinity human monocyte FcR [13], and the ability of the BR55-2 variant IgG2a

and parental IgG3 isotype to mediate tumor cell destruction when human whole blood was used as an effector system [15] suggest that these two subclasses have greater therapeutic potential in humans.

Therefore, BR55-2 subclasses IgG2a and IgG3 have been purified by chromatography from hybridoma fermentation supernatants and tested further for their ability to lyse different tumor cell lines in ADCC and CDC assays. Both were again found to be highly active, the IgG3 isotype being constantly even more active than its IgG2a counterpart in the CDC assays.

The well-known variations among different donors [13] were again observed in our experiment. The restricted binding specificity of mAb BR55-2 [21], the high cytotoxic activity of its IgG2a and IgG3 isotypes in ADCC and CDC and the ability to inhibit tumor growth in vivo [22] will be a basis for their selection as therapeutic agents. It will also be of great interest to compare, in the formal clinical trial, efficacy of both IgG3 and IgG2a isotypes of mAb BR55-2 in cancer patients since both have identical antigen avidities.

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