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Immunology

Strong antitumor activities of IgG3 antibodies to a human melanoma-associated ganglioside

(tumor antigens/immunotherapy/monoclonal antibodies/antibody-dependent cellular cytotoxicity/complement-dependent cytotoxicity)

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ABSTRACT **Three mouse monoclonal IgG3 antibodies, 2B2, IF4, and MG-21, recognize a G_{D3} ganglioside antigen that is expressed at the cell surface of most human melanomas. All three antibodies mediate antibody-dependent cellular cytotoxicity (ADCC) *in vitro* when tested with human lymphocytes as effector cells in a 2-hr or 4-hr ^{51}Cr-release test, and one antibody, MG-21, also gives strong complement-dependent cytotoxicity with human serum. Antibody 2B2, which gives ADCC also in the presence of mouse lymphocytes, inhibited the outgrowth of a human melanoma in nude mice, but antibody IF4, which showed no ADCC with mouse lymphocyte effectors, did not.**

There are many approaches to the therapeutic application of antitumor antibodies. One of the most straightforward of these is to use the antibodies alone, without further modification. However, this approach requires antibodies that have strong antitumor effects either by themselves or in the presence of complement or effector cells such as killer (K) cells or macrophages (1).

We have produced mouse monoclonal antibodies to several cell surface antigens that are primarily expressed in human melanomas (1–4). One of the most specific of these antigens is a G_{D3} ganglioside, which was first defined by Dippold and co-workers (5, 6), using their antibody R_{24}, and subsequently by Yeh and co-workers (7, 8), using an IgM antibody, 4.2. We have recently obtained three additional antibodies to this antigen, 2B2, IF4, and MG-21, which, in contrast to antibody 4.2, are of the IgG3 subclass. As described in this paper, they give strong antibody-dependent cellular cytotoxicity (ADCC) when tested against melanoma cells in the presence of human lymphocytes. Antibody MG-21, but not the others, is strongly cytotoxic to melanoma cells in the presence of human serum as a source of complement. One of the antibodies, 2B2, which gives ADCC also with mouse lymphocytes, inhibited the outgrowth of a human melanoma in nude mice.

MATERIALS AND METHODS

Tumors. Five different human melanoma lines from metastatic melanoma were used. All except one, M-2634, express high levels of the G_{D3} antigen according to binding assays, which were carried out as previously described (7, 8). Four of the lines, SK-MEL-28 (2), M-2669 clone 13, M-2634, and M-2765, were propagated *in vitro*. The fifth line, M-2586, failed to grow *in vitro* and so was serially transplanted in nude mice, where it grew better than any of the other lines.

Human lung (bronchial) carcinoma line CH27 was used as a control for antibody specificity. It does not express detectable G_{D3} antigen.

Antibodies. BALB/c mice were immunized with SK-MEL-28 cells, and their spleen cells subsequently were hybridized with NS-1 cells. Hybridoma supernatants were screened for binding to G_{D3} that had been isolated from melanoma tissue and attached to the surface of the wells of Falcon 3034 Microtest plates as previously described (7). Irrelevant gangliosides were included as controls. Hybridomas 2B2 and IF4 were derived from one hybridization, and hybridoma MG-21, from a different one. They were cloned twice by limiting dilution; all make antibodies that are IgG3 according to gel diffusion.

For comparison, two different antibodies, 96.5 and 48.7, were used. The former is directed against p97, a melanoma-associated glycoprotein of M_r 97,000 (2, 3), and the latter is specific for a proteoglycan antigen expressed by most melanomas (4). These antibodies have not, in previous (unpublished) experiments, given significant ADCC or inhibition of human melanomas in nude mice.

Antibodies were affinity-purified on a column of staphylococcal protein A covalently linked to Sepharose CL-4B (Pharmacia) by elution with 0.1 M citrate buffer, pH 3.5 or 4.5 (3).

Antibody specificity for melanoma was established by binding assays with cultured cells, as published for antibody 4.2 (7, 8). Specificity was confirmed by immunohistological studies on frozen sections (9), in which antibodies 2B2, IF4, and MG-21 stained samples from approximately 80% of metastatic melanomas, whereas normal tissues, including kidney and brain, were not stained; the specificity data for 2B2 have been published (10).

ADCC. A short-term ^{51}Cr-release test was used (11). Peripheral blood lymphocytes from five healthy human subjects were separated on Ficoll-Hypaque (12) to provide effector cells and were prescreened for low (≤5%) natural killer cell reactivity against SK-MEL-28 cells; unless indicated otherwise the ratio of lymphocytes to target cells was 100:1. Spleen lymphocytes from normal BALB/c mice were also included in two tests. Target cells (10^6) were labeled by incubation with 100 μCi (1 Ci = 37 GBq) of ^{51}Cr for 2 hr at 37°C, after which they were washed three times and resuspended in medium. The labeled cells were seeded (2×10^4 cells per well in 20 μl) into Microtiter V-bottom plates (catalog no. 1-220-25X, Dynatech Laboratories, Alexandria, VA). Purified antibody (100 μl per well) then was added, followed by 2×10^5 lymphocytes per well in 100 μl; experiments with lower numbers of lymphocytes per well and with lower antibody concentrations also were carried out, as outlined under *Results*. The mixtures were incubated for 2 or 4 hr (see Tables and text), after which the plates were centrifuged at $400 \times g$. The supernatants were removed and the radioactivity in 100-μl samples was measured with a γ-counter. There were two replicates per group; the variation between replicates was always <10%. Spontaneous release was defined as the cpm

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Abbreviation: ADCC, antibody-dependent cellular cytotoxicity.

released into the medium from target cells exposed to neither antibodies nor lymphocytes, and total release, as the number of counts released from target cells that were osmotically lysed at the end of the assay. Percent cytotoxicity was calculated as

$$\frac{\text{experimental group release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100.$$

Complement-Mediated Cytotoxicity. The ^{51}Cr-release assay also was used to test the ability of antibodies to kill melanoma cells in the presence of human serum as a source of complement. It was carried out similarly to the assays for ADCC, except that 100 μl of undiluted unheated human serum was added per microtest well, instead of a suspension of effector cells; this serum was derived from normal human subjects.

Test of Antibody Activity in Nude Mice. Athymic nude (*nu/nu*) 2- to 3-month-old male mice were obtained from Charles River Breeding Laboratories and housed in filter-top cages (five mice per cage) placed in condominium units.

The mice were grafted subcutaneously on both flanks with a small piece (1 × 1 mm diameter) of melanoma M-2586, which expresses both p97 (2) and the G_{D3} antigens (7). Each experimental group comprised 5 mice. This represented 10 "sites," since the mice were grafted on both sides. The control group had 10 mice (20 sites).

On the day after grafting and on each third day thereafter, the mice were injected via the tail vein with 1 mg of antibody in phosphate-buffered saline; this dose was chosen to provide excess antibody in the treated mice. Separate groups received antibody 2B2, IF4, or 96.5 or a combination of the three; the last group was injected with one-third of the dose of each antibody. A total of six antibody injections were given, at 3-day intervals. The control group was injected with equivalent volumes of culture medium at the same times.

The mice were inspected three times weekly over a period of 6 months. By the end of the 6 months, all the control mice had died from tumor. At each inspection, two perpendicular diameters of each palpable tumor were measured, and mean tumor diameters (±SE) were calculated; we refer to a tumor as palpable when its mean diameter is ≥2 mm. The data are presented in Table 5 as number of sites with palpable tumor per group out of the total number of sites injected.

RESULTS

ADCC. Table 1 presents an experiment in which mixtures of lymphocytes, antibodies, and ^{51}Cr-labeled target cells were incubated for 2 hr. A cytolytic effect was observed when a combination of antibody 2B2 and human effector cells was tested against SK-MEL-28 cells, which varied from 37% with antibody at 10 μg/ml to 8% at 0.01 μg/ml. CH27 cells, which lack the G_{D3} antigen, were not affected even at an antibody dose of 10 μg/ml. Antibody 2B2 alone had no significant effect, and lymphocytes alone gave only 5% cytotoxicity. The anti-proteoglycan antibody 48.7 (7) gave no ADCC. Table 1 also includes an experiment with mouse spleen cells as effectors. Antibody 2B2 gave clear-cut ADCC, whereas a different G_{D3} antibody, IF4, had only borderline activity; when combined with human lymphocytes, on the other hand, IF4 gave as good ADCC as antibody 2B2 (data not shown).

For the remainder of our experiments, the 2-hr incubation period was increased to 4 hr, since this time gives higher cytotoxicity; otherwise experiments were conducted similarly to those summarized in Table 1 and included similar controls. The findings obtained were analogous to those in Table 1 except that the ADCC effects of anti-G_{D3} antibodies on melanoma cells increased by a factor of ≈2.

To find out whether the results obtained with SK-MEL-28 could be reproduced with other melanoma lines, we tested the ability of antibody 2B2 (10 μg/ml) and human lymphocytes (100 per target cell) to give ADCC against five different melanoma lines, four of which (M-2669 clone 13, M-2765, M-2568, and SK-MEL-28) strongly display the G_{D3} antigen, and

Table 1. ADCC against SK-MEL-28 melanoma or CH27 lung carcinoma cells with human or mouse effector cells in 2-hr ^{51}Cr-release assay

	Effector cells*	Antibody (μg/ml)	^{51}Cr released,† cpm	% cytotoxicity
Experiment 1: SK-MEL-28	Human 2	2B2 (10)	22,859	37
	Human 2	2B2 (1)	19,790	31
	Human 2	2B2 (0.1)	14,151	20
	Human 2	2B2 (0.01)	7,619	8
	Human 2	2B2 (0.001)	6,070	5
	Human 2	None	6,049	5
	Human 2	48.7 (10)	2,618	0
	None	2B2 (10)	2,987	0
	None	2B2 (1)	2,842	0
Spontaneous release	None	None	3,395	
Total release			56,417	
Experiment 2: SK-MEL-28	Mouse BALB/c	2B2 (10)	11,328	21
	Mouse BALB/c	IF4 (10)	3,896	4
	Mouse BALB/c	None	2,613	1
	None	2B2 (10)	2,672	1
	None	IF4 (10)	2,742	1
Spontaneous release	None	None	2,093	
Total release			45,660	
Experiment 3: CH27	Human 1	2B2 (10)	5,090	0
	Human 1	None	6,620	0
	None	2B2 (10)	4,678	0
Spontaneous release	None	None	6,452	
Total release			81,509	

*One hundred per target cell.
†Mean of results with two replicates per group; <10% variation between replicates.

Table 2. ADCC of anti-G_{D3} (2B2 and MG-21) and control (96.5 and 48.7) antibodies as assayed against M-2669 clone 13 melanoma cells with different ratios of human lymphocytes per target cell

	% cytotoxicity with antibody at 10 or 1 μg/ml					
	2B2		MG-21		96.5	48.7
Ratio	10	1	10	1	10	10
100	68	50	82	76	0	0
10	40	17	64	52	ND	ND
1	13	7	14	13	ND	ND

Cytotoxicity was determined in 4-hr ^{51}Cr-release assay. Antibodies alone gave no cytotoxicity and lymphocytes alone gave ≥5% cytotoxicity. ND, not done.

one of which (M-2634) expresses very little (if any); the M-2568 cells were propagated as tumors in nude mice (see *Materials and Methods*). Lung carcinoma line CH27 was included as a negative control. M-2634 showed low ADCC (11%), whereas ADCC was high (65–82%) against the other four melanomas. CH27 cells were not affected.

Table 2 summarizes experiments in which two different anti-G_{D3} antibodies, 2B2 and MG-21, were tested (at 10 μg/ml and 1 μg/ml) against melanoma cells, using three different lymphocyte/target cell ratios. MG-21 gave even better ADCC than 2B2, with as much as 52% ADCC at 10 lymphocytes per target cell and 1 μg of antibody/ml.

We next evaluated the ADCC activity of antibody 2B2 in the presence of lymphocytes from five different normal donors. As shown in Table 3, effector cells from each of the donors gave strong ADCC.

The ADCC effect observed in ^{51}Cr-release tests could be competitively inhibited by adding unlabeled cells from SK-MEL-28 or M-2586 (the line used for the nude mouse experiments) but not by adding unlabeled CH27 cells (data not shown).

An experiment was done which showed that, after overnight incubation of human lymphocytes in a plastic bottle, the cells in the supernatant (not adhering to plastic) gave higher ADCC than did nonincubated cells. This suggests but does not prove that the effector cells are not macrophages.

Complement-Dependent Cytotoxicity. We studied whether antibodies 2B2 or MG-21 were cytotoxic to G_{D3}-positive melanoma in the presence of human serum as a source of complement, using a 4-hr ^{51}Cr-release assay similar to the ADCC assay but adding undiluted unheated human serum instead of effector cells. Antibody MG-21 gave a strong cytotoxic effect in the presence of human serum, whereas antibody 2B2 gave no effect (Table 4); IF4 also was negative (data not shown). CH27 lung carcinoma cells, which were used as controls, were not affected. Heat inactivation of the human serum abolished its effect.

Table 3. Antibody 2B2 (10 μg/ml) ADCC against melanoma M-2669 clone 13 with lymphocytes from normal healthy human subjects

Lymphocyte donor	% cytotoxicity with lymphocyte/target cell ratio	
	100:1	10:1
A	82	50
B	55	26
C	64	ND
D	85	ND
E	88	ND

Cytotoxicity was determined in a 4-hr ^{51}Cr-release assay. Antibody alone gave no cytotoxicity and lymphocytes alone gave ≤5% cytotoxicity. ND, not done.

Table 4. Cytotoxicity of antibody 2B2 and MG-21 in the presence of unheated human serum

Antibody	Conc., μg/ml	% cytotoxicity	
		M-2669 clone 13	M-2765
2B2	20	1	ND
	10	0	ND
MG-21	50	ND	98*
	20	86*	67*
	10	98*	ND
	5	66*	ND
	2.5	34*	ND
	1.25	27*	ND

Cytotoxicity was determined in a 4-hr ^{51}Cr-release assay. No cytotoxicity was seen with antibody alone or with human serum alone. ND, not done.
*Percentage cytotoxicity statistically different from 0, $P < 0.01$.

Antitumor Effects *in Vivo*. We tested whether antibodies 2B2 or IF4 had any antitumor effect *in vivo*. They were chosen since 2B2 but not IF4 gave ADCC with mouse effector cells. Melanoma M-2586 was used as target, since it grows extremely well in nude mice, expresses the G_{D3} antigen, can be used as target for 2B2-mediated ADCC, and can competitively inhibit ADCC against SK-MEL-28 cells. M-2586 was grafted onto both flanks of nude mice, and the mice were injected with a dose of antibody expected to give a blood concentration exceeding that needed for ADCC *in vivo*. Anti-p97 antibody 96.5 was tested in parallel, as was a combination of all three antibodies (same total antibody concentration).

As shown in Table 5, antibody 2B2 almost completely suppressed the outgrowth of melanoma grafts, with only 1 of 10 injected sites having a detectable tumor 4 months after grafting, as compared to 19 of 20 sites in the control. The small implanted tumor pieces thus had been rejected at all 10 sites in the 2B2 group except for one. Six months after grafting, 4 mice in the 2B2 group survived, tumor-free, but all the controls were dead with large (>15-mm diameter) tumors. Neither antibody 96.5 nor antibody IF4 inhibited tumor outgrowth. The group receiving a combination of antibodies 2B2, IF4, and 96.5 also showed inhibition, with 3 of 10 sites developing progressively growing tumors.

The inhibition mediated by antibody 2B2 was statistically significant from 2 months after tumors were implanted whether the comparison with the control group was made on

Table 5. Antibody 2B2 inhibits outgrowth of human melanoma in nude mice

Treatment	Sites with tumor/total number of sites	
	2 months after transplant	4 months after transplant*
Control (culture medium)	18/20	19/20
Antibody 2B2 (anti-G_{D3})	1/10	1/10
Antibody IF4 (anti-G_{D3})	8/10	10/10
Antibody 96.5 (anti-p97)	9/10	10/10
Combination (2B2, IF4, and 96.5)	3/10	3/10

A 1 × 1 mm tumor piece (human melanoma M-2586) was grafted on each flank of nude mice. The next day and at five subsequent times, at 3-day intervals, they were injected intravenously with 1 mg of antibody. There were 5 mice (10 sites) per group except for the control, which had 10 mice (20 sites).
*All mice were dead at 6 months after transplantation except for 4 of the 5 mice who received antibody 2B2 and 2 of the 5 mice who received the combination; these mice were all alive and tumor-free.

the basis of the number of sites with tumor, using a Fischer table ($P < 0.001$), or on the basis of mean tumor diameters, using Student's t test ($P < 0.001$).

DISCUSSION

We have obtained three IgG3 antibodies, 2B2, IF4, and MG-21, that bind strongly to a human melanoma-associated G_{D3} antigen. They mediate ADCC when combined with human effector cells, and one of the antibodies, MG-21, kills melanoma cells in the presence of human serum as a source of complement. Antibody 2B2, which gives ADCC also with mouse effector cells, was found to inhibit the outgrowth of a human melanoma in nude mice. Although most antitumor antibodies tested by us and others lack these characteristics, an antibody to a proteoglycan antigen of melanoma cells, described by Schultz *et al.* (13), has an antitumor effect that appears similar to that of 2B2.

High cytolytic activity was detected after 2 hr in ^{51}Cr-release assays when human lymphocytes were combined with antibody and tested against cells expressing large amounts of the G_{D3} antigen; lymphocytes or antibodies alone were ineffective. Significant ADCC could be seen even at an antibody dose of 10 ng/ml and with one lymphocyte per target cell. The ADCC effect was antigen-specific, since cells lacking the G_{D3} antigen were not killed and the effect could be competitively inhibited by addition of antigen-positive tumor cells.

Antibody 2B2 gave significant ADCC also with mouse spleen lymphocytes as effectors, but antibody IF4 did not. This provided an impetus to investigate the antitumor activity of the two antibodies in mice grafted with a human melanoma expressing high levels of the G_{D3} antigen. Antibody 2B2 effectively prevented tumor outgrowth in most of the treated mice, whereas antibody IF4 gave no tumor inhibition. However, we cannot conclude from the present data that the *in vivo* effect of antibody 2B2 operates via ADCC; it could conceivably have other mechanisms, including direct antibody effects on tumor cells (14) and macrophage activation.

Since antibody 2B2 could destroy small (1 mm diameter) tumor implants in nude mice and its ADCC activity was greater in the presence of human rather than mouse lymphocytes, one may speculate that it also might destroy small melanoma implants (in the form of micrometastases) in man. This, if true, may have therapeutic applications in patients who have deeply penetrating primary melanomas and, therefore, poor prognosis. Studies on patients with advanced melanoma (as normally chosen for pilot trials) are complicated by the fact that lymphocytes from most such patients do not give detectable ADCC with any of our antibodies (unpublished results). The therapeutic (and other) implications of the apparent ability of antibody MG-21 to activate human complement need to be investigated.

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