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Human Monoclonal Antibody to Ganglioside GD2-Inhibited Human Melanoma Xenograft*

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Abstract—*A human IgM monoclonal antibody was produced* in vitro *against OFA-I-2, a human tumor membrane antigen. This antigen is expressed on tumors of neuroectodermal origin, and has been identified as the ganglioside GD2. This study examines the anti-tumor effect of the monoclonal antibody against a GD2-positive human melanoma cell line, M14, inoculated subcutaneously into athymic CD-1 nude mice. Tumor-free survival was prolonged markedly when the monoclonal antibody and M14 cells were inoculated simultaneously. When antibody and complement were also injected into established tumor nodules, M14 tumor growth was suppressed. However, intraperitoneal injection of the antibody did not alter the growth of the subcutaneously inoculated M14 cells. The antibody has no effect on the growth of a GD2-negative melanoma cell line, M24. These results indicate that the human monoclonal antibody to GD2 may be useful for the suppression of GD2-positive tumor cells in cancer patients if the tumor can be directly exposed to the antibody and complement.*

INTRODUCTION

WITH THE development of the hybridoma techniques, the use of specific anti-tumor antibody for passive immunization became more practical. Monoclonal antibodies have suppressed the growth of transplanted tumors in syngeneic mice [1, 2] and caused a complete remission in a patient with B lymphoma [3]. However, such studies have been limited to murine monoclonal antibodies. Although Schlom *et al.* [4] described human monoclonal antibody production by hybrids of human lymphocytes and mouse myeloma, none of the investigators have succeeded in establishing human-to-human hybridomas that produce antibodies specific for human tumors.

Recently, we successfully established a human B lymphoblastoid cell line, L72, that produces IgM monoclonal antibody to a tumor-associated antigen (OFA-I-2) [5]. The anti-OFA-I-2 antibody specifically reacts with malignant cells of neuroectodermal origin, including melanoma, glioma and neuroblastoma, but not with other types of human tumors or normal adult cells [5]. Chemically, the antigen was identified as ganglioside GD2 [6]. The antibody is highly cytotoxic to GD2-positive tumor cells *in vitro* in the presence of complement. In the present study we tested its cytotoxic effect on OFA-I-2-positive melanoma cells grown in athymic nude mice in order to investigate the potential usefulness of the antibody as an anti-tumor reagent for cancer patients.

MATERIALS AND METHODS

Mice

Four- to six-week-old male CD-1 athymic nude mice (nu/nu) were purchased from the Charles River Breeding Laboratories, Inc., North Wilmington, MA. Mice were maintained in a pathogen-free environment and none accidentally died during the experiments.

Tumor cells

Human melanoma cell lines M14 and M24 were selected on the basis of their transplantability and growth patterns after subcutaneous injection of tumors in CD-1 nude mice. M14 and M24

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displayed similar growth patterns. M14 was known to be GD2-positive whereas M24 was negative for GD2 [5]. Both cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Cells were collected when they were 50% confluent, usually 3–5 days after passage in tissue culture. The cells were treated with trypsin and washed with RPMI 1640. The cells were then inoculated subcutaneously into CD-1 nude mice according to the experimental protocol.

Human monoclonal antibody (anti-GD2)

An EBV-transformed human lymphoblastoid cell line (L72) that produced IgM κ-type monoclonal antibody against the ganglioside GD2 was established from PBL of a melanoma patient [5]. Briefly, the IgM fraction [antibody(Ab)-positive fraction] was collected from the antibody-producing L72 spent medium using salt and hypotonic precipitations and ultracentrifugation. The Ab-positive fraction usually consisted of 70% IgM monoclonal antibody and 30% FCS-derived proteins. The monoclonal character of the immunoglobulin produced by the L72 cell line was determined by agarose isoelectric focusing and immunofixation techniques [7]. The protein concentration of the fraction was measured by the Coomassie blue dye-binding assay [8] and the IgM concentration was measured by radioimmunoassay [9].

In this study we used an 'anti-GD2 negative fraction' as a control. The fraction was obtained as follows: after several months in continuous culture, L72 often lost its ability to produce anti-GD2. However, if this Ab-negative spent medium was concentrated and partially purified by the same method as that for the Ab-positive medium, a minimum amount of human IgM became detectable in the radioimmunoassay. The amount was usually 1 μg or less/ml. The concentration of FCS-derived proteins was adjusted to that of the Ab-positive fraction in each experiment. As a control, this Ab-negative fraction eliminated the effect of FCS-derived proteins on tumor growth. We also used non-specific human IgM (100 μg) from a patient with macroglobulinemia as another control.

Cytotoxicity assay

Complement-dependent cytotoxicity was assayed by trypan blue exclusion. Fifty microliters of tumor cell suspension adjusted to 2×10^5/ml, 50 μl of anti-GD2 in serial dilution and 50 μl of four-fold diluted fresh rabbit serum used as complement sources were mixed and incubated at 37°C for 90 min. Dead cells were determined after the addition of 150 μl of 0.5% trypan blue dye solution.

Experimental protocol

(a) To determine the direct cytotoxic effect of anti-OFA-I-2 antibody on M14 melanoma cells *in vivo*, melanoma cells (M14 and M24) were suspended in RPMI 1640 at various concentrations. Aliquots of 0.1 ml containing 2×10^5 cells and 0.1 ml of the Ab-positive fraction were mixed and injected subcutaneously into the anterior thoracic region of CD-1 nude mice [10]. After tumor inoculation mice were examined twice a day for the detection of visible tumor nodules. Measurements of the nodules were recorded. Time from tumor injection to the appearance of visible tumor was expressed as tumor-free interval. (b) To determine the anti-tumor effect of systemic injection of anti-OFA-I-2 antibody, 0.2 ml of tumor cell suspension (2×10^5 cells) was injected subcutaneously into the anterior thoracic region immediately after the intraperitoneal injection of the Ab-positive or -negative fraction (0.2 ml). (c) To determine the anti-tumor effect of intraregional injection of anti-OFA-I-2, 1×10^6 M14 or M24 cells were inoculated subcutaneously into CD-1 nude mice. Ten days after inoculation, mice bearing similar-sized tumors were selected and randomly divided into five groups. The following antibody fractions (0.1 ml) were injected into each tumor of the five groups: group I: Ab-negative fraction; group II: rabbit complement alone; group III: Ab-negative fraction and rabbit complement; group IV: Ab-positive fraction; group V: Ab-positive fraction and rabbit complement.

RESULTS

Tumor growth after subcutaneous inoculation of various concentrations of M14 and M24 melanoma cells

M14, a GD2-positive human melanoma cell line, and M24, a GD2-negative cell line, were selected for their transplantability in CD-1 nude mice. Various numbers of viable melanoma cells were injected subcutaneously in the anterior thoracic region. The minimal cell number required for 100% growth was determined as 2×10^5 cells for both of M14 and M24. Tumor-free intervals were 12 and 9 days respectively. All animals were observed for at least 3 months, after which time they were killed by cervical dislocation. The liver, lungs, kidneys, spleen, pancreas and regional and mediastinal lymph nodes were searched for indications of metastases. No mascroscopic metastases were observed in any of the animals.

Complement-dependent cytotoxicity in vitro

Complement-dependent cytotoxicity of the anti-GD2 monoclonal antibody was assayed *in vitro* against the M14 and M24 target cells. Because the expression of GD2 on M14 differed slightly depending upon culture conditions, the mean % cytotoxicity (2×10^5 cells) ranged from 72 to 95% with 25 μg of antibody and rabbit complement in seven independent experiments. Table 1 displays representative data. When M24 acted as the target cell, no amount of antibody (100 μg) and complement had a cytotoxic effect in any of the seven independent assays.

*Table 1. Complement-dependent cytotoxicity of monoclonal anti-GD2 against GD2-positive (M14) and -negative (M24) human melanoma cells**

Antibody (μg/ml)†	Rabbit complement‡	% cytotoxicity of target cells (mean ± S.D.) M14	M24
0	-	4 ± 1	5 ± 1
0	+	5 ± 1	4 ± 1
100	-	6 ± 2	4 ± 2
100	+	97 ± 1	8 ± 2
50	+	97 ± 1	7 ± 2
25	+	95 ± 2	5 ± 1
10	+	85 ± 2	6 ± 1
1	+	24 ± 5	5 ± 1

*50 μl of 2×10^5/ml cells were each mixed with an equal volume of antibody and complement in the assay. Results were expressed as mean % cytotoxicity ± S.D. of five samples.

†Purified antibody.

‡Fresh rabbit whole serum diluted 1:4 was used as complement source.

Effect of simultaneous administration of anti-OFA-I-2 and tumor cells on in vivo *tumor growth suppression*

The mixture of 2×10^5 M14 cells and the IgM fraction prepared from anti-GD2 monoclonal antibody-producing spent medium were injected subcutaneously into the anterior thoracic region of the CD-1 mice. An Ab-negative fraction prepared from spent medium of cells not producing anti-GD2 was tested as a control. The tumor-free interval was recorded as a function of days after tumor inoculation. When M14 were injected together with the Ab-negative fraction, the mean tumor-free interval was 12 days (range 9–15). When M14 cells were mixed with the Ab-positive fraction, the mean tumor-free interval was significantly longer and increased with an increasing concentration of antibody, as shown in Table 2. A statistically significant prolongation in tumor-free interval was obtained when the antibody dosage was 25 μg or greater. However, complete suppression of tumor growth did not occur from dosages ranging from 1 to 100 μg.

The possibility that human IgM might have a direct cytotoxic effect against the M14 cells was eliminated by the fact that 100 μg of IgM from a macroglobulinemia patient's serum had no tumor-suppressive effect.

In order to clarify the specificity of anti-GD2 for GD2-positive tumor cells, the GD2-negative melanoma cells (M24) were tested in the same manner using 100 μg antibody. There was no significant difference in tumor-free interval between the Ab-positive and -negative fraction-treated groups of mice (Table 2).

*Table 2. Tumor-free interval of CD-1 nude mice inoculated with M14 or M24 and various dosages of monoclonal antibody**

Cells admixed with:	IgM (μg)	No. of mice tested	Tumor-free interval (mean)
M14:			
Anti-OFA-I-2	100	4	12–52 (33.0)‡
	50	9¶	12–45 (30.2)‡
	25	4	12–26 (19.5)§
	10	3	8–21 (16.0)
	1	3	9–15 (12.3)
Anti-OFA-I-2-negative fraction†	<0.1	11¶	9–15 (11.8)
IgM myeloma serum	100	4	8–14 (12.8)
M24:			
Anti-OFA-I-2	100	4	8–11 (9.0)
Anti-OFA-I-2-negative fraction	<0.1	4	8–11 (9.5)

*Each mouse was injected with the mixture of 2×10^5 M14 or M24 and antibody subcutaneously at the anterior lateral thoracic wall.

†Anti-OFA-I-2-negative fraction was collected from antibody-non-producing L72 spent medium described in the Materials and Methods section.

‡$P < 0.001$.

§$P < 0.05$.

¶The result of the experimental group using 50 μg of IgM anti-OFA-I-2 and that of the control group using anti-OFA-I-2-negative fraction were obtained from two separate experiments. Other groups were obtained from one experiment.

It is known that mouse complement activity is usually low when compared with that of the rabbit or the human. Therefore M14 cells were mixed with anti-GD2 (50 μg) and 1:2 diluted rabbit complement at 4°C *in vitro* before the mixture was injected subcutaneously into the CD-1 nude mice. Cell viability was maintained in all of the four groups before injection. Visible tumor was not apparent in 2/5 mice (40%) for more than 3 months (Fig. 1).

The effect of systemic administration of monoclonal anti-GD2 antibody on subcutaneous tumor growth

Anti-GD2 (100–300 μg/mouse) was injected intraperitoneally just before the M14 cells alone were inoculated subcutaneously. Intraperitoneal injection rather than intravenous injection was chosen for systemic injection of antibody and complement because (1) it was technically easy; (2) a large volume could be injected; and (3) a rapid penetration of human IgM antibody and complement into circulation could be achieved. No significant prolongation of tumor-free interval was apparent in the antibody-injected group when it was compared with the control group (Fig. 2). A mixture of M14 and rabbit complement was inoculated subcutaneously, and again there was no significant prolongation of tumor-free interval.

Effect of intratumor injection of monoclonal anti-GD2 and complement

Ten days after subcutaneous inoculation the tumor nodules from the M14 and M24 inoculations were 0.3–0.4 cm in diameter. Each tumor nodule was then injected with 0.1 ml of the various antibody fractions. Tumor size (length and width) was measured by a vernier caliper every 2 days. Table 3 shows the tumor size on day 14 post-intratumor injection. The growth of M14 nodules receiving the Ab-positive fraction was slightly suppressed when compared with nodules receiving the Ab-negative fraction ($P < 0.01$). M14 nodules injected with antibody and complement were significantly suppressed ($P < 0.001$), whereas antibody and complement had no tumor-suppressive effect on M24 nodules.

DISCUSSION

The athymic nude mouse is a valuable tool for the *in vivo* study of human neoplasms. Because it lacks thymic epithelium and has a deficient immune response, the mouse readily accepts human tumor transplants. Several reports have shown that these transplanted human tumors preserve their biological and biochemical characteristics and are sensitive to chemotherapeutic [11, 12] as well as immunologic manipulation [13, 14]. In this study the *in vivo* effects of anti-GD2 was assessed on human melanomas transplanted in nude mice.

Tumor-free survival was prolonged markedly when the monoclonal antibody and GD2-positive M14 cells were inoculated simultaneously (Table 2 and Fig. 1). The prolongation of tumor-free interval is the result of specific lysis of the GD2-positive M14 cells by anti-OFA-I-2 and endogenous mouse complement. The possibility that human IgM could non-specifically destroy human melanoma cells is negligible, because no effect was found when the GD2-negative M24 tumors were injected with anti-GD2, or when non-specific human IgM from a macroglobulinemia patient's sera was combined with

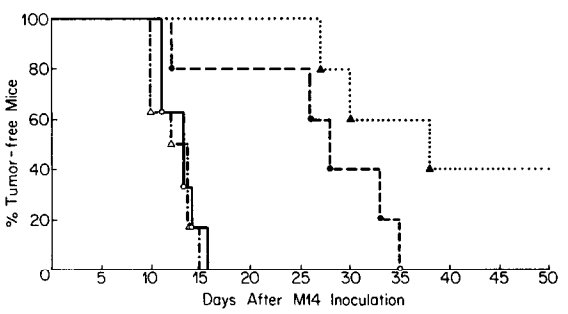

Fig. 1. Tumor-free interval of CD-1 nude mice inoculated with M14, anti-OFA-I-2 and rabbit complement. Male mice were injected with a mixture of 2 × 10⁵ M14 and Ab-negative fraction (○——○ 6 mice), M14, Ab-negative fraction and complement (△·–·–·△ 6 mice), M14 and Ab-positive fraction (●– – –● 5 mice), or M14, antibody-positive fraction and complement (▲·····▲ 5 mice).

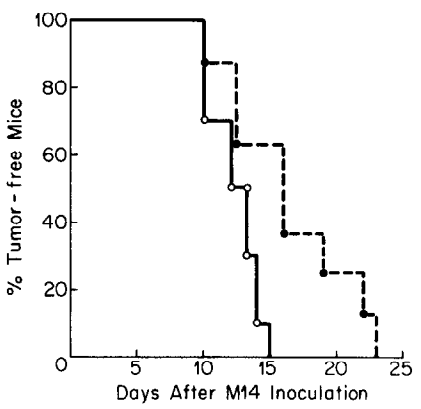

Fig. 2. The effect of systemic injection of human monoclonal antibody to OFA-I-2 on subcutaneous growth of M14 in CD-1 nude mice. Male mice were injected with 2 × 10⁵ M14 cells subcutaneously immediately after the intraperitoneal injection of Ab-negative fraction (○——○ 10 mice, <0.1 μg IgM/mouse) or Ab-positive fraction (●– – –● 8 mice, 200 μg IgM/mouse).

*Table 3. Suppression of tumor growth by intratumor injection of antibody to GD2 in subcutaneous nodules of M14 and M24**

Intratumor injection of:	IgM (μg)	Tumor size ($L \times W$ cm^2: mean ± S.D.) M14 (5 mice) 0 days	M14 (5 mice) 14 days	M24 (3 mice) 0 days	M24 (3 mice) 14 days
Antibody-negative fraction†	<0.1	0.14 ± 0.03	0.78 ± 0.13	0.16 ± 0.05	0.90 ± 0.10
Rabbit complement	0	0.14 ± 0.03	0.81 ± 0.11	0.17 ± 0.03	0.94 ± 0.11
Antibody-negative fraction and rabbit complement	<0.1	0.14 ± 0.03	0.73 ± 0.12	0.16 ± 0.04	0.87 ± 0.05
Antibody-positive fraction	200	0.13 ± 0.04	0.50 ± 0.13‡	0.16 ± 0.04	0.87 ± 0.05
Antibody-positive fraction and rabbit complement	200	0.14 ± 0.04	0.27 ± 0.13§	0.16 ± 0.03	0.90 ± 0.10

*10^6 M14 or M24 cells were inoculated subcutaneously in CD-1 nude mice. Ten days after tumor inoculation each of the tumor nodules received 0.1 ml of various kinds of fractions (0 days). Fourteen days after the injection of antibody the tumor size was calculated from its length and width (14 days).

†Anti-GD2-negative fraction was collected from antibody-non-producing L72 spent medium described in the Materials and Methods section.

‡$P < 0.01$.

§$P < 0.001$.

GD2-positive M14 cells (Table 2). The possibility that anti-GD2 might stimulate an endogenous mouse immune response was ruled out for the following reasons. The tumor-free interval was not prolonged when M24 cells were injected subcutaneously with anti-OFA-I-2 or when anti-GD2 was administered intraperitoneally. In addition, when M14 was inoculated subcutaneously with and without anti-GD2 on opposite sides of the thoracic wall, the tumor-free interval was significantly prolonged at the site of the M14 and anti-GD2 injection but not at the site of M14 alone (data not shown). It is also possible that anti-GD2 acted by exerting an effect at the level of the cell membrane [15], because anti-GD2 reacts specifically with a membrane antigen, GD2. However, this explanation is unlikely because when M14 cells were cultured with anti-GD2 *in vitro* for 5 days in the absence of complement, proliferation was slightly enhanced in the anti-GD2-added wells but no growth inhibition was seen (data not shown). Although anti-GD2 did not cause complete rejection of tumor growth, it did evoke a prolonged tumor-free interval. Further studies with modified nude mouse systems may provide additional information on its potential for human application. One modification could be to heighten the complement system. When whole sera from CD-1 nude mice were used as the complement source in the *in vitro* antibody-dependent complement-mediated cytolysis assay, complement activity was extremely low when compared to rabbit or human complement. In Fig. 1 it was shown that when M14 cells were inoculated with anti-GD2 and rabbit complement, 2/5 mice were free of visible tumor for more than 3 months. When antibody was injected into already established tumor nodules, M14 tumor growth was significantly suppressed only under the coexistence of anti-GD2 and rabbit complement (Table 3).

Our previous studies using the immune cytotoxicity test and polyclonal serum antibodies to OFA-I suggested that OFA-I could be modulated if tumor cells were incubated with antibody [16]. In these studies we did not distinguish anti-OFA-I-2 (anti-GD2) from anti-OFA-I-1. Therefore we were unable to distinguish which antibody modified the antigen. However, in our preliminary experiments with the purified monoclonal anti-GD2 no significant antigenic modulation was observed.

In syngeneic mouse systems, passive immunotherapy has been effective when the transplanted tumor has metastasized or when tumor burden is small, and when the monoclonal antibody was given either simultaneously with the tumor transplantation or soon thereafter [1, 17, 18]. However, in our study the systemic injection of monoclonal antibody failed to suppress subcutaneous tumor growth (Fig. 2). The subcutaneously transplanted melanomas appeared to be well circumscribed by a capsule of dense connective tissue [19]. This fibrous capsule was detected as early as day 1 post-transplantation. The ineffectiveness of passive immunotherapy by the intraperitoneal route might be a result of this characteristic of the subcutaneously transplanted human melanoma cells, because the fibrous capsule may prevent an effective accumulation of the antibody in the tumor nodules. It is also possible that IgM may not be able to penetrate into extravascular spaces. In an attempt to show that IgM anti-GD2 antibody administered intraperitoneally did not localize on subcutaneously inoculated M14 cells, we performed the following experiment (data not shown). The antibody was injected intraperitoneally immediately after M14 cells were inoculated intraperitoneally or subcutaneously in CD-1 nude mice. The binding of the antibody to M14 was then measured by the immune adherence assay *in vitro*. Antibody

localization was only detected on the intraperitoneally inoculated M14 cells. Subcutaneously inoculated M14 cells formed rosettes with human erythrocytes only after antibody and guinea pig complement were added to the assay. These findings indicate that intraperitoneally administered antibody can access intraperitoneally inoculated M14 but not subcutaneously inoculated M14. In any case, administration of the anti-OFA-I-2 did not enhance tumor growth.

These results indicate that anti-GD2 may be useful for the suppression of GD2-positive tumor cells if tumor can be directly exposed to the antibody and complement, such as for hematogenous metastases. Recently, Miller and coworkers reported on results of experimental mouse monoclonal antibody therapy in a lymphoma patient [3]. They used a total dosage of 500 mg of antibody to achieve an effective result in a patient with B-cell lymphoma. In our previous study with stages II and III melanoma patients we found a significant prolongation of disease-free interval in those patients with a high titer of IgM anti-OFA-I antibody in their serum [20]. The antibody titer was greater than 80% by the indirect membrane immunofluorescence test and greater than 32 IA_{50} in the immune adherence (IA) assay. According to our calculations using purified monoclonal anti-OFA-I-2 antibody, approximately 100 ng/ml IgM anti-OFA-I-2 was equivalent to an IA_{50} of 1; thus more than 20 mg of antibody would be required to maintain an IA_{50} of 32 in the serum of an adult male, assuming no circulating OFA-I-2 antigen is present. As previously reported, only 2–3 mg of pure IgM antibody were recovered from 1 l of L72 spent medium at a recovery rate of 75% [7]. Although anti-OFA-I-2 could be collected in unlimited amounts from these cultured cells, the yield does not seem to be practical for the purpose of cancer therapy in man. Therefore a crucial requirement at this stage of anti-GD2 antibody development is a method for large-scale production.

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