HYBRIDOMA AND HYBRIDOMICS
Volume 21, Number 6, 2002

In Vivo and *In Vitro* Anti-Tumor Effect of 14F7 Monoclonal Antibody

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ABSTRACT

The 14F7 monoclonal antibody (MAb) is an IgG_1 antibody that reacts specifically with GM3 (NeuGc) and with tissue sections of human tumors. We demonstrated here that this MAb is agglutinin that specifically agglutinated horse erythrocytes. Additionally, the capacity of 14F7 MAb to mediate cytotoxicity against GM3 (NeuGc)-positive murine myeloma cells, *in vitro* and *in vivo*, was evaluated. High concentrations of 14F7 MAb were needed to induce complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) against the murine myeloma cells. The most relevant finding was the ability of this MAb to directly kill the target cells without participation of complement. This cytotoxicity was dependent on the temperature and MAb concentration and the number of the target cells. *In vivo*, the passive treatment with 14F7 MAb produced a strong anti-tumor activity, similar to the anti-tumoral response obtained with standard chemotherapy treatment.

INTRODUCTION

CHANGES IN THE GANGLIOSIDE EXPRESSION between tumors and normal tissues have been described.[1] In Oncology, monoclonal antibodies (MAbs) against gangliosides have gained clinical interest due to their effect in the induction of regression of cutaneous melanoma and metastases of neuroblastoma.[2–5]

Complement and cellular-dependent cytotoxicity have been reported as possible effector anti-tumor mechanisms for anti-ganglioside antibodies.[6,7] In addition, it has been proposed that the role of IgM anti-ganglioside antibodies is the abrogation of the immunosuppression induced by gangliosides shed from tumor.[8]

The *N*-glycolylated form of sialic acid (NeuGc) is not expressed in human normal tissues.[9,10] In contrast, the expression of NeuGc has been detected on tumor cells by monoclonal or polyclonal antibodies of chicken, human or murine origin.[11–13] Additionally, increased levels of NeuGc in human breast tumors were detected by biochemical methods.[14] These findings make this antigen a potential target for immunotherapy.

We recently reported the generation of a murine IgG1 MAb, named 14F7, highly specific against GM3(NeuGc) that strongly recognized human melanoma and breast tumors.[15] In this paper, we demonstrate that 14F7 MAb is an inefficient antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) mediator but, this MAb has the outstanding property of causing cell death independent of complement in GM3(NeuGc) bearing murine tumor cells. Also, we show that the treatment of tumor-bearing mice with 14F7 MAb induces a strong anti-tumor activity.

MATERIALS AND METHODS

Animals

Balb/c, C57BL/6 and nu/nu NMRI mice, 6 to 8 weeks old, were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). Mice were housed in plastic cages under standard conditions with access to rodent chow and water *ad libitum*. Animal care was in accordance with our institutional guidelines.

MAbs

14F7 MAb (IgG1, κ) was generated by immunization of Balb/c mice with GM3 (NeuGc) ganglioside hydrofophically conjugated with human very-low-density-lipoproteins(VLDL).[15] P3 MAb

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Table 1. Hemagglutination Capacity of 14F7 MAb

Monoclonal antibody	Erythrocyte source			
	Horse	Dog	Human	Monkeys
14F7 (4°C)	+++	−	−	−
14F7 (37°C)	+++	−	−	−
P3 (4°C)	−	−	−	−
P3 (37°C)	−	−	−	−

Scored based on the MAb required for HA.
+++ = 0.5–2 μg/mL; − = >100 μg/mL.

was used as controls. P3 MAb is (IgM, κ) that reacts with a broad battery of NueGc sialic acid containing-gangliosides.[14]

Cells

Tumor cell line. The murine tumor cell lines used were P3 X63. Ag8. 653 (myeloma), B16-F10 (melanoma) and 3LLc (lung carcinoma). Cells were grown in RPMI-1640 medium (Gibco, Gaithersburg, MD), supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin. Prior to mice injection, B16-F10 and 3LLc cells were trypsinized and P3 X63 Ag8. 653 were resuspended by pipetting. Cells were washed and resuspended in phosphate-buffered saline (PBS), pH 7,4. Tumor cells were counted and viability was greater than 90%, as assayed by trypan blue exclusion test. Only, the myeloma cells were positive for the expression of GM3 (NeuGc) and for the recognition by 14F7 MAb, as assessed by TLC-immunostaining, immunohistochemistry, and flow cytometry analysis.

Erythrocytes. Horse, dog, monkey, and human erythrocytes were obtained, washed and resuspended at 2% v/v in 0.9% sodium chloride. Among them, only those of horse origin expressed GM3 (NeuGc).[16]

Hemagglutination assay

The hemagglutination assay was performed as previously reported.[17] Briefly, various concentrations of 14F7 or P3 MAbs (1–100 μg/mL) were mixed with 2% erythrocyte suspensions in round-bottomed well microplates. Then, the plate was incubated 1 h on ice or at 37°C. The end point was scored as the lowest MAb concentration that produced erythrocyte agglutination.

In vitro *citotoxicity assays*

CDC assay was performed as described.[18] Briefly, P3 X63 Ag 8. 653 myeloma cells were labeled with ^{51}Cr (Amersham, Laboratories, Buckingham, UK) for 2 h in serum-free medium at 37°C, followed by three washes. The target cells (10^4 cell/well) were incubated with different concentrations of 14F7 MAb (spontaneous release is measured by incubation of the target cells with medium alone). P3 MAb, which also recognizes the myeloma cells in flow cytometry experiments, was used as control antibody. The target cells (10^4 cell/well) were incubated with different concentrations of 14F7 MAb (10 and 50 μg/mL) for 30 min at 37°C in the presence or absence of rabbit complement (CENPALAB, Havana, Cuba). Plates were centrifuged at 500 × g for 5 min and the supernatant (100 μL) was collected and radioactivity counted in a gamma-scintillation counter. The percentage of specific cytotoxicity was calculated as follows: % cytotoxicity = 100 × (experimental release-spontaneous release) / (maximum release-spontaneous release), where the maximum release was produced by incubation of the target cells with 10% Triton X-100 and spontaneous release is that measured by incubation of the target cells with medium alone. As control antibody was used P3 MAb, which also recognize the myeloma cells in flow cytometry experiments.

ADCC was done, as previously reported.[19] Lymphocytes harvested from spleen of BALB/C and C57BL/6 mice were used as effector cells. The murine myeloma cells were labeled with ^{51}Cr, as described above, and used as target cells. Targets were incubated with 50–100 μg/mL of 14F7 MAb for 30 min at 37°C, and washed. Labeled target cells, treated with or without 14F7 MAb, were incubated with various numbers of effector cells for 18 h at 37°C. The same control MAb used in CDC experiments was also included. The percentage of lysis was calculated as described before.

Also, the effect of 14F7 MAb on the viability of P3X63 Ag8 653 myeloma cells was studied by flow cytometry using the propidium iodide (PI) exclusion assay.[20] Different number of myeloma cells (2×10^5—10^6/100 μL) were mixed with 50 μL of different concentrations of 14F7 MAb (0.01–1 mg/mL). Cells were incubated for 30 min at 4°C and then the cells were washed with PBS, pH 7.4. Finally, cells were resuspended in 600 μL of PBS and 10 μL of PI (0.2 mg/mL) (Sigma, St. Louis, MO) was added. The analysis was performed by flow cytometry using fluorescent activated cell analyzer (Cytoron, Ortho Diagnostic System, Raritan, NJ). P3 (an IgM MAb, which recognizes the same antigen) MAb was used as control antibody.

In vivo *effect of 14F7 MAb on tumor cell growth in mice*

Two different types of experiments were performed. (a) Different murine solid tumors, expressing or not GM3 (NeuGc) were used. With P3 X63 Ag8 653 myeloma cells, to obtain solid tumors, the cells were inoculated in nu/nu NMRI mice ($n = 5$) and the animals received 1×10^6 myeloma cells intramuscularly (i.m.) (Day 0). On Days 0, 2, 4, 6, 8, and 10, mice were injected intraperitoneally (i.p.) with 200 μg of 14F7 MAb in PBS. Similar experiments were performed using C57BL/6 mice ($n = 10$) inoculated with 2×10^3 B16-F10 cells or 5×10^4

Table 2. Complement-dependent Cytotoxicity Mediated by 14F7 MAb

MAb (μg/mL)		% of lysis	
		Rabbit serum dilution 1:5	1:10
14F7	50	51	45
	10	4	2
P3	50	65	65
	10	62	61

TABLE 3. CYTOTOXICITY INDUCED WITHOUT COMPLEMENT

		Cell concentration		
MAb (mg/mL)	*Target cells*	*10^7 cells/mL (%)*	*0.5×10^7 cells/mL (%)*	*10^6 cells/mL (%)*
P3 (0.1)	P3 × 63 Ag8 653 myeloma	5.9[a]	ND	ND
P3 (0.01)	P3 × 63 Ag8 653 myeloma	4.5	ND	ND
14F7 (1)	P3 × 63 Ag8 653 myeloma	80	36	5%
14F7 (0.4)	P3 × 63 Ag8 653 myeloma	63	ND	ND
14F7 (0.1)	P3 × 63 Ag8 653 myeloma	62	10	6%
14F7 (0.01)	P3 × 63 Ag8 653 myeloma	7	6	5.9%

[a]Percent of dead cells measured using the PI exclusion assay. ND: not determinated.

3LLc. Also, a group of nu/nu NMRI mice were inoculated with the myeloma cells, but the treatment with 14F7 MAb started on Day 10.

(b) Balb/c mice ($n = 10$) were injected i.p. with 0.5 mL of incomplete Freund¥s adjuvant. After 3 days, animals were inoculated ip with 10^4 myeloma (Day 0). Animals were treated i.v. on Days 0, 2, 4, 6, 8, and 10 with 100 or 200 μg of 14F7 MAb. Animals that were injected iv with 20 mg/kg of cyclophosphamide (Lemery S.A., Mexico, D.F., Mexico) weekly were used as the positive control group.[21] Mice that received PBS instead of the MAb were used as negative controls.

Statistical analysis. The two largest perpendicular diameters of tumors were recorded with a caliper, twice a week to evaluate tumor growth. Analysis of variance (ANOVA) test was used to compare mean tumor volumes among each group. Survival was analyzed by Kaplan–Mayer method, and groups were compared by log–rank test, $p < 0.05$ was considered as significant and $p < 0.01$ as highly significant.

RESULTS

The specific hemagglutination capacity of 14F7 MAb is shown in Table 1. 14F7 MAb was able to agglutinate horse erythrocytes, but not those from dog, monkey, or human origin. The difference among these different types of erythrocytes is that in horse erythrocytes, 95% of the lipid containing sialic acid is in the form of GM3 (NeuGc)[16]; while this ganglioside is absent in the other erythrocyte species studied. The agglutination was observed both at 37°C and at 4°C. Although, P3 MAb recognizes GM3 (NeuGc), it was not able to agglutinate horse erythrocytes.

14F7 MAb incubated with myeloma cells in the presence of rabbit complement was tested for its ability to lyse these GM3 (NeuGc)-positive cells. Table 2 shows the results of this experiment. The incubation for 30 min at 37°C with 50 μg/mL of 14F7 produced 50 and 45% cytotoxicity of the target cells, depending on the rabbit complement dilution used. Lower concentration of 14F7 MAb (10 μg/mL) did not produce cytotoxicity. P3 MAb was able to produce more than 60% of lysis at both concentrations.

To assess whether 14F7 could induce ADCC, P3 X63 Ag8 653 myeloma cells were labeled with ^{51}Cr and incubated in the presence of the MAb and the effector cells. 14F7 MAb induced lysis of target cells equal or higher than 30% only when the ratio of mouse splenocytes:target cells was 80:1 and when MAb was used at a concentration of 100 μg/mL (Table 3). The control P3 MAb produced similar cytotoxicity effect than 14F7 MAb. No cytolytic effect was detected when target cells were incubated only in the presence of the antibody. The percent of ^{51}Cr release by effector cells alone (without the antibody), was always between 1–10%.

In the propidium iodine cytotoxicity exclusion assay performed at 4°C for 30 min, the percent of myeloma cells in the PI+ population (dead cells) correlated directly to 14F7 MAb and target cell concentrations, and not with P3 MAb concentration. The best cytotoxicity with 14F7 MAb was induced at the concentration of 10^7 myeloma cells/mL (Table 4).

The role of 14F7 MAb in modulating the growth of GM3 (NeuGc)-positive and -negative tumors was studied (Fig. 1). In the experiment nu/nu NMRI mice were inoculated on day 0

TABLE 4. ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY MEDIATED BY 14F7 MAb AGAINST MURINE MYELOMA CELLS

		(%) of Lysis C57BL6 splenocytes E/T ratio[a]			*(%) of Lysis Balb/c splenocytes E/T ratio*		
MAb	*μg/mL*	*20:1*	*40:1*	*80:1*	*20:1*	*40:1*	*80:1*
14F7	50	12.6	24.1	26.7	8.3	27.8	36.8
14F7	100	7.5	26.6	42.1	9.6	28.3	44.8
P3	50	8.8	11.5	21.5	8.9	22.2	39.4
P3	100	4.5	22	38	11	25.2	39

[a]E/T ratio: effector:target cells ratio.

with 1×10^6 murine myeloma cells and were treated iv with 14F7 MAb or with PBS on Days 0, 2, 4, 6, 8, and 10. A significant suppression of tumor growth was observed in the mice that received passive therapy with 14F7 MAb, in comparison with the control group ($p < 0.01$). In contrast, when the treatment with the MAb started 10 days after the inoculation of the myeloma cells, no differences were observed when compared with the control mice (Fig. 1B). No differences were detected in tumor growth between 14F7 MAb-treated and PBS-treated C57BL/6 mice inoculated with B16-F10 or 3LLc tumor cells (antigen negative) (Figs. 1C and D).

Balb/c mice bearing P3 X63 Ag8 653 ascitic tumors were kept until moribund in order to study the effect of the passive treatment with14F7 MAb on animal survival, in comparison with the effect produced by chemotherapy treatment with cyclophosphamide as positive control. Figure 2 shows the results of this experiment. The survival of tumor-bearing animals was significantly prolonged in the groups of animals treated i.v. with 100 μg ($p = 0.007$) or 200 μg ($p = 0.003$) of 14F7 MAb, in comparison with the group that received only PBS. On the other hand, no differences in the prolongation of survival were observed among the groups treated with 14F7 MAb and the ones treated with cyclophosphamide ($p = 0.05$).

DISCUSSION

In a previous study, we described that 14F7 MAb binds specifically to GM3 (NeuGc), and that it also reacts with human breast and melanoma tumors; in contrast, with its low reactivity in normal tissues.[15] In this study, the anti-tumor activity of 14F7 MAb against murine tumor cells expressing GM3 (NeuGc) was observed and the possible effector mechanisms through which this antibody exert its biological effect was analyzed.

CDC and ADCC are effector mechanisms that have been suggested to be involved in the antibody effect against some tumors expressing gangliosides.[22,23] Nevertheless, with 14F7 MAb high concentrations of the antibody are needed to induce

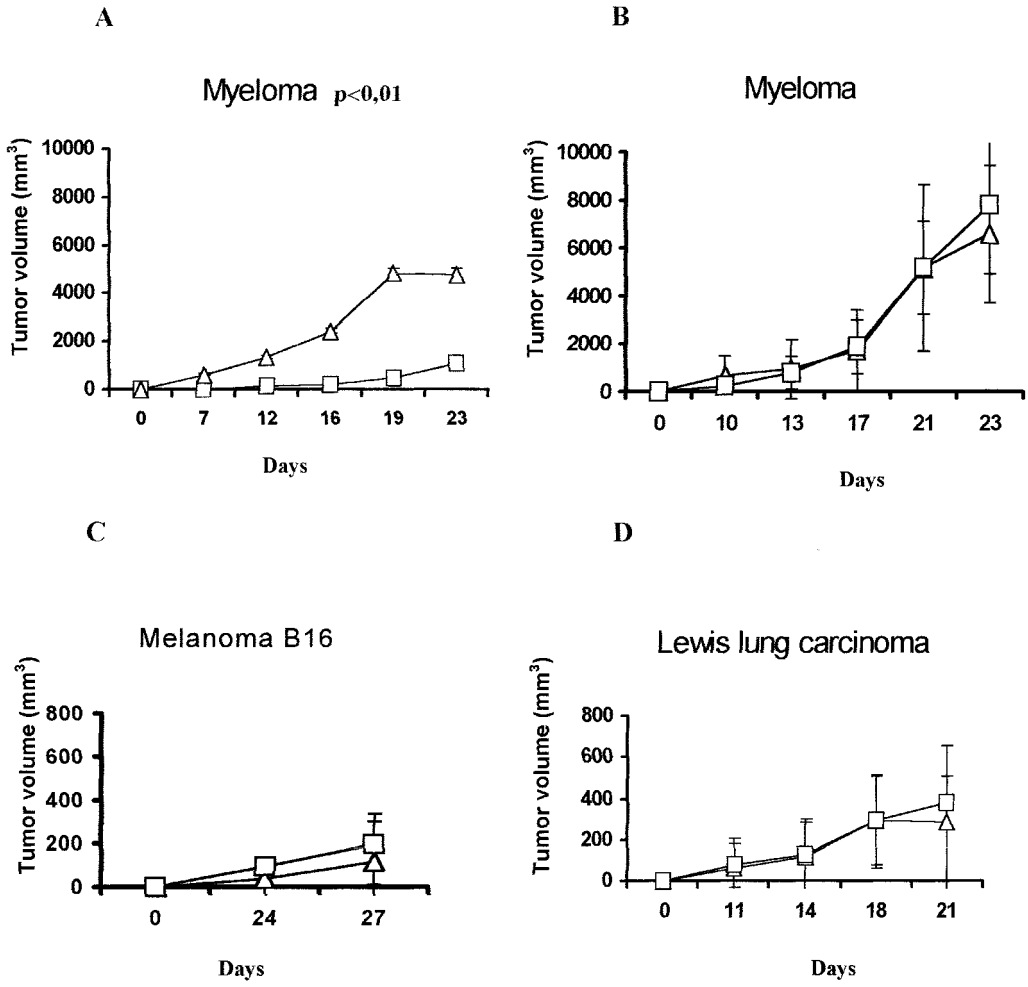

FIG. 1. Effect of 14F7 MAb on murine tumor growth. Mice were treated with 14F7 MAb or with PBS (control) (**A**), (**C**), and (**D**) represent the tumor growth in mice treated with 14F7 MAb on days 0, 2, 4, 6, 8 and 10. (**B**) shows the results obtained when the treatment with 14F7 MAb started on Day 10. Data represent means ± SEM. $p < 0.01$, ANOVA test.

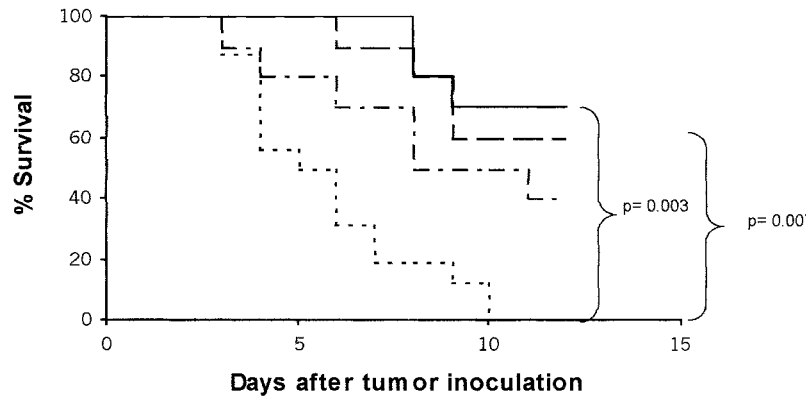

FIG. 2. Survival of 14F7-treated mice. Treatments were as follows: ———100 μg/mL of 14F7 MAb, —— 200 μg/mL of 14F7 MAb, —-— 200 μg of cyclophosphamide and ----PBS. Comparison of overall survival between the different groups was tested by Log–rank test.

cytotoxic effects by both mechanisms against myeloma cells, in spite off their high expression of GM3 (NeuGc). Thus, 14F7 MAb is an inefficient ADCC and CDC mediator. This result is in agreement with the fact that murine IgG_1 antibodies are weak inducers of CDC and ADCC.[24]

On the other hand, 14F7 MAb showed a strong anti-tumor activity against the myeloma cells *in vivo*. The weak CDC and ADCC activity could be involved, but another possible explanation for this phenomenon is the characteristic of this MAb to produce a complement independent cytotoxicity at low temperature. Apoptosis does not seem to be involved in the loss of cellular viability since time to cell death is short not corresponding with the duration of events in the programmed cell death mechanism. This uncommon property was previously described for other MAbs. One of them, named RE2, was found while searching for a xenogeneic antibody specific for mouse T-cell membrane molecules. This antibody at 4°C produced a complement-independent lysis of a T-cell clone used for MAbs screening.[25] The other examples were human VH4-34 gene-derived anti-i cold agglutinin (CA) MAbs that bound human and murine B cells.[26] The binding of B cells by these MAbs led also to a rapid noncomplement dependent cytotoxicity at 4°C. Similar to these MAbs, the cytotoxicity produced by the incubation of target cells with 14F7 MAb is dependent on the temperature and MAb concentration, but not on complement activity.

The mechanisms by which 14F7 induces cell death are still unknown, but as was proposed for RE2,[26] a cross-linking of GM3(NeuGc) molecules on the surface of target cells by 14F7 MAb might induce complement-independent cell lysis.

It is noteworthy that P3 MAb, an IgM antibody that also recognizes GM3(NeuGc), was incapable of agglutinating horse erythrocytes. While, 14F7 MAb, an IgG_1 antibody, was able to agglutinate these erythrocytes at 37°C and at 4°C. Interestingly, the VH4-34 antibody is a cold agglutinin that also induces complement independent cell death.[26]

Growth inhibition and prolonged survival of the myeloma tumor are evidences of anti tumor effect obtained after treatment with 14F7 MAb. This result correlated with the high expression of GM3 (NeuGc) ganglioside as target molecule, shown by biochemical and immumohistochemistry analysis in this murine myeloma cell line. Furthermore, no anti-tumor effects were observed with the antigen negative cell lines, the antigen negative murine tumors LLc and B16.

In this study we demonstrate that when nu/nu NMRI mice were treated ip with 14F7 MAb the same day of myeloma cell inoculation, a suppression of tumor growth was observed; but when the MAb treatment started 10 days after challenge, no anti-tumor effect was observed. This result indicates that the use of 14F7 MAb could be effective mainly in an adjuvant therapy, as has been proposed for other MAbs that react with other tumor-associated antigens.[4]

Therapy with cyclophosphamide produces tumor regression in murine myelomas.[21] Our results show that treatment with 14F7 MAb produced an increase in anti-tumor effect related to MAb dose similar to the cyclophosphamide chemotherapy. Nevertheless, optimization of the administration frequency and MAb concentration should be performed.

New clinical evidences of anti tumor effect of MAbs in cancer patients have appeared in the last years.[27,28] Most MAbs developed for treatment of solid tumors are indicated combined with chemotherapy or radiotherapy. The evidences of anti-tumor effect shown here with 14F7 MAb in experimental solid tumors indicate its potential use for passive immunotherapy in these tumor types.

A phase I tumor imaging clinical trial with 14F7 MAb in advanced cancer patients will be carried out in the near future in order to define the capacity of this MAb to target tumors *in vivo*, as a critical result to use it in passive immunotherapy.

ACKNOWLEDGMENTS

The authors thank Dr. Franz Torres for statistical advice, technician Dariel Morales for valuable support and supervision at the animal care facility; and Dr. Blanca Rosa Tormo for their helpful advice and for editorial assistance. Financial support

was provided in part by Laboratorios ELEA (Buenos Aires, Argentina) and Cuba Government.

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Received for publication September 4, 2002. Accepted for publication September 20, 2002.