Antitumor properties of an anti-idiotypic monoclonal antibody in relation to N-glycolyl-containing gangliosides

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Abstract. We examined the antitumor effects of 1E10 monoclonal antibody, an anti-idiotypic IgG to an IgM monoclonal antibody, named P3, that reacts specifically with N-glycolyl-containing gangliosides and also recognizes antigens in human breast and melanoma tumors. Two murine tumor cell lines positive for the P3 antibody, F3II mammary carcinoma (BALB/c) and B16 melanoma (C57BL/6), were employed. In BALB/c mice, vaccination with several i.p. doses at 14-day intervals of 50 μg of 1E10 coupled to keyhole limpet hemocyanin in Freund's adjuvant, significantly reduced s.c. tumor growth of F3II carcinoma cells and the number of spontaneous lung metastases. Also, the effect of 1E10 as a biological response modifier on tumor lung colonization was evaluated in C57BL/6 mice injected i.v. with B16 melanoma cells. Interestingly, i.v. administration of 10 μg of uncoupled 1E10 antibody, 10 to 14 days after inoculation of B16 cells, dramatically reduced the number of experimental metastases in comparison with lungs from mice treated with an irrelevant IgG. The present data suggest that this "non-internal image" anti-idiotypic monoclonal antibody may activate more than one mechanism of antitumor response against melanoma and mammary tumor cells.

Introduction

One strategy to generate an effective immune response against tumor-associated antigens (TAAs) involves the use of anti-idiotype antibodies (Ab2) as antigen surrogates. The use of Ab2 as vaccines was suggested on the basis of Jerne's theory (1), that postulates the existence of Ab2 that carry the "internal image" of antigenic epitopes. This capacity to mimic a TAA (β-type Ab2) has been considered an important characteristic to take into account when selecting an anti-idiotype antibody to induce antitumor immunity (2,3). In fact, different Ab2 monoclonal antibodies (MAbs) that mimic tumor-associated gangliosides, such as GM3, GD3 and GD2, have been obtained (4-9), and some of them are being evaluated in clinical trials to treat cancer patients (10-12).

We have previously generated and characterized an Ab2 murine MAb to a murine Ab1 MAb named P3, which recognizes specifically N-glycolyl sialic acid on several monosialo- and disialogangliosides, and also reacts with antigens expressed in human melanoma and breast tumors (13,14). The IgG1 Ab2 MAb obtained, designated 1E10, was able to block the binding of P3 MAb to GM3 (NeuGc) ganglioside and induced anti-anti-idiotypic antibodies (Ab3) in syngeneic animals. These Ab3 antibodies generated by 1E10 MAb were characterized to bear P3 MAb idiotopes, but 1E10 MAb failed to elicit autologous Ab3 antibodies with the same specificity than P3 MAb. Thus, 1E10 MAb is not an "internal image" antibody and was classified as γ-type Ab2 (15).

Although Ab2 bearing the internal image of TAAs are considered excellent candidates to be selected for immunotherapy, several reports demonstrated that the induction of antigen-specific humoral immune response due to the immunization with β type Ab2 was not predictive of the biological effect induced by the antibody (16, Köhler et al, Proc 8th Int Congr Of Immunology, pp619-626, Budapest, 1992). The selection of an Ab2 MAb to be used in immunotherapy should be based in its biological effect, rather than in its classification as α, β or γ (17). For this reason, we studied the effect of our "non-internal image" Ab2 following two different approaches: as a classical anti-idiotypic vaccine in a syngeneic model and as a biological response modifier in an allogeneic model. In the present study we demonstrate that the treatment of tumor-bearing mice with 1E10 MAb induce a strong antitumor activity.

Materials and methods

Animals. C57BL/6 and BALB/c inbred mice, with an age of 8-14 weeks and a weight of 20-25 g, were purchased from the Center for Laboratory Animal Production (CENPALAB, Havana, Cuba) and from the Animal Care Division of the Institute of Oncology Angel H. Roffo (Buenos Aires, Argentina). Mice were housed in plastic cages under standard conditions with access to rodent chow and water ad libitum.

MAb. 1E10 anti-idiotype MAb (IgG1, κ) was generated against the murine anti-N-glycolyl-containing gangliosides P3 MAb (IgM) (15). Ior C5 MAb, a murine MAb that recognizes an antigen preferentially expressed in human colorectal cells (18), was used as isotypic and allotypic control. MAbs were purified from ascitic fluid by affinity chromatography using a Protein A-Sepharose column (Amersham Pharmacia Biotech).

Cell lines. B16 murine melanoma cell line (ATCC Number CRL-6322, subline F0) was kindly provided by Dr. O. Podhajcer (Campomar Foundation, Buenos Aires, Argentina). B16 cells were grown in RPMI-1640 (Gibco BRL) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. The sarcomatoid mammary carcinoma cell line F3II is a highly invasive and metastatic variant established from a clone of a spontaneous, hormone-independent BALB/c mouse mammary tumor (19). F3II cells were maintained in minimal essential medium 41500 (MEM) (Gibco BRL) supplemented with 5% FCS. For injection into mice, cell suspensions were prepared by detaching the cells from culture flasks with trypsin-EDTA (0.05%), followed by washing and resuspension 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. Both cell lines were positive for P3 (Ab1) MAb, as assessed by indirect immunoperoxidase staining on tumor cell monolayers or paraffin-embedded subcutaneous tumor sections.

Induction of Ab3 response in mice. For induction of Ab3 response, BALB/c mice were injected i.v. with 10 μg of purified 1E10 MAb. Mouse serum samples were obtained before receiving each dose and 7-14 days after. Mice treated in the same way, but without 1E10 MAb, were used as controls.

Preparation of F(ab')2 fragments. MAb F(ab')2 fragments were obtained using a procedure previously described (20). Briefly, MAb was incubated in 2 mM citrate buffer, pH 3.5, in the presence of pepsin (Sigma Chemical Co.) at 37^{o}C, in a proportion enzyme:MAb of 1:20. The reaction was stopped after 4 h by rising the pH up to 8 with 2M Tris solution. The Fc fragments and the non-digested immunoglobulin were removed by adsorption to Protein A-Sepharose (Amersham Pharmacia Biotech), and the non-adsorbed fraction was eluted with 1.5 M Glycin 3M NaCl buffer, pH 8.9. This fraction was extensively dialyzed with PBS and concentrated at least to 1 mg/ml. F(ab')2 fragments were purified by gel filtration chromatography using Superdex-75 column (Amersham Pharmacia Biotech) equilibrated with PBS, pH 7.4. The purity of the fragments was confirmed by SDS-PAGE in 7.5% gels.

Antibody binding assay. An ELISA assay was used to measure Ab3 reactivity in mouse sera. Solid-phase ELISA was performed using 96-well polystyrene microtiter plates (High binding, Costar), which were coated with 10 μg/ml of 1E10 F(ab')2 fragments and incubated overnight at 4^{o}C. After washing with PBS containing 0.05% Tween 20, the plates were blocked for 1 h at room temperature with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (BSA). Then, the diluted serum samples were added, the plates were incubated 2 h at 37^{o}C and washed again. The second antibody, alkaline-phosphatase-conjugated goat anti-mouse IgG or IgM (Fc fragment specific) (Sigma), was added and after an incubation of 1 h at 37^{o}C, the plates were washed and the reaction was developed with p-nitrophenylphosphate substrate (Sigma) in dietanolamine buffer, pH 9.8. Absorbance was measured at 405 nm in an ELISA reader (Organon Teknike). Ior C5 F(ab')2 fragments were used as controls. Three samples of each experiment were tested and the standard deviation was less than 10% for all values. Background values of absorbance were less than 0.1.

T cell proliferation assays. BALB/c and C57BL/6 mice were immunized once by s.c. injection at the base of their tails with 100 μg of uncoupled 1E10 MAb in complete Freund's adjuvant. Ten days later, lymph node cells were collected and seeded into 96-well plates at a density of 3-5 x 10^5 cells/well, and incubated for 54 h with different concentrations of 1E10 MAb (80 – 2.5 μg/ml). Then, the cells were pulsed with 1 μCi/well of ^{3}H-thymidine (Amersham) for 18 h. Thymidine uptake was determined by scintillation counting. Ior C5 was used as an isotypic and allotypic MAb control. Concanavalin A (80 μg/ml) was used as positive control. Tests were run in triplicates. Stimulation index was calculated for each triplicate by dividing the mean radioactivity of stimulated cells with that of unstimulated cells. A stimulation index greater than 3 was considered positive. Similar experiments using lymph node cells from naive BALB/c mice were also performed.

Flow cytometry analysis. Binding of 1E10 MAb to B16 cells was analyzed by flow cytometry. Briefly, B16 cells incubated with purified 1E10 MAb were washed and subsequently incubated with FITC-conjugated goat anti-mouse immunoglobulins (Dakoppatts) at a dilution 1:100. After washing, the cells were resuspended and analyzed on a fluorescent-activated cell analyzer (Becton-Dickinson). A murine anti-GM3 MAb was used as a positive control.

Antitumor experiments. Two different types of experiments were done:

a) BALB/c mice were immunized i.p. 7 times at 14 days intervals with 50 μg of 1E10 MAb coupled to keyhole limpet hemocyanin (KLH), the antibody 1E10 alone, or the carrier KLH alone, in 0.1 ml of PBS, mixed with an equal volume of complete (first dose) or incomplete (next doses) Freund's adjuvant (Sigma). Control animals received only the immunological adjuvant mixed with PBS. For tumor cell challenges, F3II cells were injected in the subcutis of the right flank. Seven days after the third immunization (designated as day 0 of the experiment) each mouse received 2 x 10^5 live mammary tumor cells in 0.2 ml of culture medium. The time of appearance of local tumors was monitored by palpation and further confirmed by histopathology. The mean of the two largest perpendicular diameters was recorded with a caliper twice a

week to evaluate tumor growth. At day 60, mice were sacrificed by cervical dislocation and necropsied, according to a previous described method (21,22). To investigate the presence of spontaneous metastases, lungs were removed, fixed in Bouin′s solution, and the number of surface lung nodules was determined under a dissecting microscope. The liver, kidney, spleen and bone marrow were also removed for histological examination.

b) C57BL/6 mice were inoculated i.v. with 2.5 x 10^4 B16 cells in 0.1 ml of PBS. After 10 or 14 days, the animals were injected i.v. with 10 μg of 1E10 MAb, with an irrelevant MAb or with PBS alone. Mice were killed on day 20 after tumor inoculation, lungs were removed, fixed in Bouin's solution and lung nodules were counted.

Results

Effects of immunization with 1E10-KLH on s.c. mammary tumor growth, invasion, and spontaneous metastasis. Groups of 9-10 mice were injected i.p. 7 times with 1E10-KLH, 1E10 alone, KLH alone, or the immunological adjuvant alone (PBS control). Three doses were administered before (on days -35, -21, and -7) and 4 doses after (on days +7, +21, +35, and +49) the s.c. inoculation of F3II mammary tumor cells. Tumor incidence was 100% and tumor latency was similar in all groups (7 ± 3 days; mean ± SEM). In control animals, tumors grew by invading the muscular and adipose layers of the subcutis. At day 30, tumor cells also invaded the dermis and the dermal papillae, causing necrosis in the epidermal layer and visible ulceration on top of the tumors. As shown in Figure 1, immunization with 1E10-KLH significantly decreased subcutaneous tumor growth. No differences were observed in tumor growth between mice treated with the MAb or the carrier protein alone, in comparison with the control group.

Several subcutaneous tumors from each group were examined by routine histopathology at the end of experiment and all F3II tumors were diagnosed as spindle-cell carcinomas, as reported (19,21,22). A mild, non-significant antiinvasive effect was observed in mice immunized with 1E10-KLH (Table I). Some tumors from animals vaccinated with 1E10-KLH grew by filling the subcutis, without signs of active invasion of dermis.

Immunization with 1E10-KLH significantly reduced the spontaneous dissemination to lungs of F3II mouse mammary carcinoma cells ($p<0.05$). Interestingly, treatment with the carrier KLH alone enhanced the number of metastatic lung nodules (Figure 2). Extrapulmonary tumor colonies were not found in any of the control or immunized mice. We observed a marked splenomegaly in F3II-bearing mice in all treatment groups. The histopathological study of spleens revealed massive myelopoiesis and megakaryopoiesis. Extramedullary myelopoiesis was also evident in clusters around sinusoids in the liver of tumor-bearing mice. Bone marrow had increased cellularity (100%) and myeloid to eritroid ratio (10:1), associated with a peripheral neutrophilia.

Effects of i.v. administration of 1E10 on B16 experimental lung metastases. Seven experiments were carried out to evaluate the effect of 1E10 MAb on the lung colonization by B16 cells in C57BL/6 mice. 1E10 MAb (10 μg) injected i.v. 10 or 14 days after tumor cell inoculation significantly reduced the number of lung metastases ($p<0.001$) compared with mice administered with PBS. Figure 3 shows the pooled results of two experiments where animals were treated with the anti-idiotype MAb 14 days after tumor inoculation and sacrificed on day 20. There were not significant effects on the number of lung nodules in animals injected with the irrelevant IgG1 MAb compared to PBS controls (data not shown). Lungs from C57BL/6 inoculated with B16 cells and treated with a single injection of 1E10 MAb 10 days later are shown in Figure 4. A lower number of metastases can be observed in lungs from animals treated with the anti-idiotype MAb in comparison with those from mice treated with the IgG1 irrelevant MAb or with PBS. Table II shows the frequency distribution of metastases in animals treated or not with 1E10 Mab. In 44 out of 62 mice (71%) treated with the Ab2 MAb, less than 10 lung nodules per animal or complete regression of tumor lesions were observed.

Sera from C57BL/6 and BALB/c mice treated i.v. with a single injection of 10 μg of 1E10 MAb were analyzed by an indirect ELISA against F(ab')2 fragments of the anti-idiotypic Mab. No reactivity of the mouse sera against 1E10 was detected.

In order to define whether 1E10 MAb can induce proliferation of lymphocytes to its idiotype, lymph node cells from naive mice or from animals immunized with 1E10 MAb were challenged in vitro with the anti-idiotype MAb or the irrelevant antibody, and the

proliferative responses of the cells were measured. Neither the cells from non-immunized animals nor from 1E10 immunized mice produced any in vitro proliferative response after challenge with different concentrations of the anti-idiotype MAb.

To determine if 1E10 MAb can recognize directly murine B16 melanoma cells, the reactivity of the anti-idiotype MAb with the cells was analyzed by immune flow cytometry. As shown in Figure 5, 1E10 MAb did not show any binding to B16 cells, whereas the anti-GM3 control MAb strongly stained the cells.

Discussion

In the present study we have evaluated the capacity of the "non-internal image" 1E10 Ab2 MAb to induce antitumor effects in murine tumor models.

The results show that the immunization of syngeneic mice with 1E10 MAb coupled to KLH and emulsified in Freund's adjuvant significantly reduced subcutaneous tumor growth of F3II carcinoma cells and blocked local invasion of muscular and adipose layers of the subcutis and dermis in some animals. Furthermore, immunization with 1E10-KLH also decreases the number of spontaneous lung metastases. All these data indicate that 1E10 Ab2 MAb antibody could activate an antitumor response against F3II mammary tumor cells and induce a significant delay in the progression of the disease.

In all groups, progression of subcutaneous F3II mammary tumors induced a massive myelopoiesis in bone marrow and spleen. We have reported previously that secretion of ectopic cytokines such as granulocyte-macrophage colony-stimulating growth factor (GM-CSF) by F3II carcinoma cells stimulates myelopoiesis and also may play an important role in tumor progression and metastasis formation (23). In another mammary tumor model, production of GM-CSF-like activity, also regulated the metastatic potential (24). In addition, tumor-derived GM-CSF was able to induce myelopoiesis and immunosuppression, modulating tumor growth in vivo (25).

Previous analysis of the antibody response in BALB/c mice immunized with the Ab2 coupled to KLH and in the presence of adjuvant, showed that a strong Ab3 response specific to 1E10 MAb was produced. These Ab3 antibodies were characterized to share idiotypes with P3 MAb (Ab1), but they did not have the same specificity against

gangliosides of P3 MAb (11). Nevertheless, we can not discard the possibility that the vaccination with 1E10 MAb can induce the production of antibodies that recognize antigens others than N-glycolylated gangliosides in the tumors.

The antitumor effect of 1E10 MAb was also demonstrated in assays evaluating experimental metastases in allogeneic animals, where mice were not vaccinated but treated i.v. with a single low dose of the Ab2 MAb alone. In those experiments, a significant reduction of the number of B16 melanoma metastatic lung nodules was observed. The fact that the treatment with 1E10 MAb was delayed at least 10 days after B16 tumor cells were injected, suggests that the MAb has effect in tumors that are already established in the lungs.

A similar antitumor activity was reported previously for an Ab1 MAb, called BAT, generated against Daudi human B lymphoblastoid cell line, that recognizes B16 melanoma cells. BAT binds and activates T cells, and its antitumor activity was related to its immunostimulatory properties (26,27). In contrast, 1E10 MAb was unable to induce in vitro proliferation of lymph node lymphocytes from either naive mice or animals that were injected with 1E10 MAb emulsified in Freund´s adjuvant.

On the other hand, Ab3 antibodies were not detected in the sera of mice treated i.v. with one dose of 10 μg of 1E10 MAb alone. This result suggests that Ab3 antibodies do not seem to be responsible of the antimetastatic effect observed in mice inoculated with mouse melanoma B16 cells. It seems unlikely that the Ab2 exerts a direct effect against the tumor because no recognition of B16 cells by 1E10 MAb, measured by FACS, was observed. All these data suggested that the antimetastatic effect observed after the intravenous treatment with 1E10 MAb was not mediated by the variable region repertoire. Nevertheless, it seems clear that 1E10 MAb act as a biological response modifier when it is administered to tumor-bearing mice.

The mechanisms through 1E10 MAb exerts its antitumor effects are still unknown. Further investigations will be directed to analyze the effect of 1E10 MAb on NK and NKT cells due to their importance as effector cells against tumors (28-30). Despite the mechanism involved, it is clear that 1E10 MAb antibody could activate an antitumor response and induce a significant effect in tumor or metastatic progression.

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Table I. Effect of immunization with 1E10-KLH on the local invasiveness of F3II primary tumors.

Treatment [a]	Local invasion [b] [Positive animals/total (%)]	
	Muscular and adipose layers of the subcutis	Underlying flank musculature
PBS Control	9/9 (100)	2/9 (22)
1E10-KLH	7/9 (77)[c]	3/9 (33)
1E10	10/10 (100)	1/10 (10)
KLH	10/10 (100)	6/10 (60)[c]

[a] Mice were injected i.p. 7 times with 1E10-KLH, 1E10 alone, KLH alone, or only the immunological adjuvant (PBS control), as described in 'Materials and methods'.

[b] F3II mammary tumor cells were inoculated s.c. on day 0. At day 60, animals were necropsied and tumor specimens were used for histopathological examination.

[c] Non-significant, Fisher's exact Test.

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Table II. Effect of i.v. administration of 1E10 MAb on the frequency distribution of experimental lung metastases produced by B16 cells.

Treatment [a]	Number of lung metastases per mouse [b] [Number of animals/total (%)]			
	None	1-10	11-50	>50
PBS Control	0/55 (0)	17/55 (31)	17/55 (31)	21/55 (38)
1E10 MAb	11/62 (18) [c]	33/62 (53)	7/62 (11)	11/62 (18)

[a] Mice were injected i.v. with 10 μg of 1E10 MAb or PBS 10-14 days after tumor cell inoculation, as described in 'Materials and methods'.

[b] B16 melanoma cells were inoculated i.v. on day 0. At day 20, mice were killed and lung nodules were counted.

[c] $p<0.001$, Kolmogorov-Smirnov Test.

Legend to Figures

Figure 1. Tumor response after immunization with 1E10-KLH in female inbred mice inoculated with F3II mammary tumor cells. Mice were injected i.p. 7 times with 1E10-KLH, 1E10 alone, KLH alone, or the immunological adjuvant alone (PBS control), as described in 'Materials and methods'. Values represent means ± SEM from at least 9 animals. *$p<0.05$, ANOVA test.

Figure 2. Effect of immunization with 1E10-KLH on spontaneous lung metastases produced by F3II cells. Mice were injected i.p. 7 times with 1E10-KLH, 1E10 alone, KLH alone, or only the immunological adjuvant (PBS control) as described in 'Materials and methods'. The number of spontaneous lung metastases was determined 60 days after s.c. inoculation of F3II mammary tumor cells in female inbred mice. Each value represents the number of metastases counted in the lungs of each mouse, and arrows denote the medians. *$p<0.05$, Kruskal-Wallis test.

Figure 3. Inhibition of experimental lung metastases by 1E10 MAb. Results are based in two experiments in which B16 cells were inoculated i.v. at a concentration of 2.5×10^4 cells. Mice were injected 14 days after tumor cell inoculation with 10 μg of purified 1E10 MAb or PBS alone. Animals were killed on day 20. Each value represents the number of metastases counted in the lungs of each mouse, and arrows denote the medians. *$p<0.05$, Mann-Whitney test.

Figure 4. Inhibition of experimental lung metastases by 1E10 MAb. Mice were injected 10 days after B16 melanoma cell inoculation with 10 μg of purified 1E10 MAb or an irrelevant MAb, as described in 'Materials and methods'. Lungs from animals treated with PBS (A), ior C5 MAb (B), and 1E10 MAb (C) are shown.

Figure 5. 1E10 MAb tested on B16 melanoma cell line by flow cytometry analysis. B16 cells were incubated with 1E10 MAb, with an anti-GM3 MAb (positive control), or with

PBS (negative control). The reaction was developed with goat anti-mouse immunoglobulins-FITC labeled.