

An Anti-Idiotypic Vaccine Elicits a Specific Response to *N*-Glycolyl Sialic Acid Residues of Glycoconjugates in Melanoma Patients¹

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We generated the 1E10 γ -type anti-idiotypic mAb (Ab2) specific to an Ab1 mAb able to react specifically with *N*-glycolyl-containing gangliosides and with Ags expressed on human melanoma and breast carcinoma cells. This Ab2 mAb induced an Ab response in animal models sharing immunochemically defined idiotopes with the Ab1. The treatment of tumor-bearing mice with 1E10 mAb induced a strong antitumor activity. A clinical trial was conducted in 20 patients with advanced malignant melanoma. Patients were treated with six intradermal injections of aluminum hydroxide-precipitated 1E10 anti-Id mAb given at 2-wk intervals. Sixteen of the 17 patients who received at least four doses of the anti-Id vaccine develop Ab3 Abs capable of inhibiting Ab2 binding to Ab1 (Ab3Id+). In contrast to the incapacity of 1E10 mAb to generate Ab3 Abs with the same antigenic specificity as the Ab1 mAb in mice, a very specific and strong Ab3 response against *N*-glycolyl-containing gangliosides was induced in 16 patients (Ab3Ag+). No evidence of serious or unexpected adverse effects has been observed in this clinical trial. 1E10 anti-Id vaccine was safe, well tolerated, and immunologically effective, with most patients being able to generate a specific immune response against 1E10 and Neu-glycolyl-GM₃ ganglioside. *The Journal of Immunology*, 2002, 168: 2523–2529.

Tumor-associated gangliosides are carbohydrate self-Ags that are very poorly immunogenic. Different approaches to improving the immune response to ganglioside vaccines in cancer patients have been conducted by several groups. Purified gangliosides attached to bacillus Calmette-Guérin or coupled to a carrier protein such as keyhole limpet hemocyanin and whole cell vaccines have been able to induce an Ab response in vaccinated patients (1–3).

One strategy for inducing humoral and cellular immune response against gangliosides involves the use of anti-Id mAbs (Ab2 mAbs) as Ag surrogates. Ab2 mAbs that mimic gangliosides highly expressed on tumor cells, such as GD3 and GD2, have been obtained, and they have the property to elicit circulating Abs specific to the corresponding ganglioside when they are injected into syngeneic or xenogeneic animals (4–6). Encouraging results have been obtained in clinical trials where Ab2 mAbs mimicking gangliosides have been used as vaccines to treat cancer patients (7–11).

Neu-glycolyl (NeuGc)³-containing gangliosides are not expressed in normal human tissues, but their presence in several human tumors has been reported, making them ideal targets for cancer immunotherapy (12–15).

We generated a mouse IgM κ mAb identified as P3 (Ab1), which recognizes selectively NeuGc-containing gangliosides and Ags expressed in human breast tumors (16, 17). In addition, the reactivity of P3 mAb with human melanoma biopsies has been demonstrated (Fig. 1). By immunizing BALB/c mice with P3 mAb we have obtained an anti-idiotypic (Ab2) mAb, named 1E10. This IgG1 Ab2 mAb was able to block the binding of the Ab1 mAb to NeuGc-GM₃ ganglioside. 1E10 mAb produced strong anti-anti-idiotypic Ab (Ab3) response in syngeneic mice and induced Abs bearing P3 mAb idiotopes, but it failed to elicit Ab3 Abs with the same Ag specificity as P3 mAb. These results demonstrated that this Ab2 mAb is not an internal image Ab and may be classified as γ -type Ab2 mAb (18). Previous results showed that the treatment of tumor-bearing mice with this “noninternal image” Ab2 induces a strong antitumor activity (19).

In this work we report the results of the use of 1E10 anti-Id mAb for treatment of patients with advanced malignant melanoma. Our results provide evidence that the immunization of the patients with 1E10 mAb is safe and induces not only a specific anti-Id response but also Ag-specific Ab3 responses in patients.

Materials and Methods

Gangliosides

Gangliosides NeuAc-GM₃, NeuGc-GM₃, GM₁, GD_{1a}, and NeuGc-GM₂ were provided by Dr. L. E. Fernández (Vaccine Department, Center of Molecular Immunology, Havana, Cuba). NeuAc-GM₃ and NeuGc-GM₃ gangliosides were purified from dog and horse erythrocytes, respectively, as described earlier (20). GM₁ and GD_{1a} gangliosides were isolated and purified from bovine brain (21). NeuGc-GM₂ was purified from mouse liver (22). NeuAc-GM₂ obtained from salmon liver was kindly supplied by Dr. J. Portoukalian (Glycobiology Laboratory, Faculty of Medicine Lyon-Sud, Lyon, France). GD3 from bovine brain was purchased from Sigma-Aldrich (St. Louis, MO). Homogeneity and purity of gangliosides is >95% as determined by TLC and densitometry (23).

Anti-idiotypic Ab (1E10) for the clinical trial

1E10 Ab2 mAb (IgG1, κ) was generated by immunizing BALB/c mice with P3 mAb (IgM, κ) (16) as previously described (18). 1E10 mAb was purified

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³ Abbreviations used in this paper: NeuGc, Neu-glycolyl; HPTLC, high-performance thin layer chromatography.

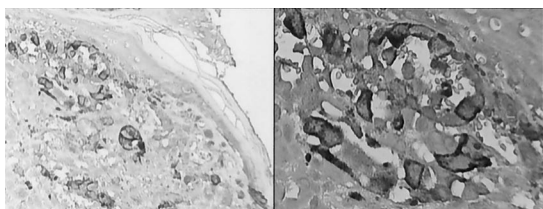


FIGURE 1. Immunohistochemical staining of melanoma cells using P3 mAb (Ab1). Melanoma sections were incubated with P3 mAb and the reaction was developed by the avidin-biotin complex technique. Original magnification: *left*, $\times 100$; *right*, $\times 400$.

from ascites in the Good Manufacturing Practice facilities of the Center of Molecular Immunology. Purification of 1E10 mAb was performed by DEAE-exchange chromatography followed by affinity chromatography on protein A-CL Sepharose 4 B column and size exclusion chromatography using Sephadex G-25 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated Ig ($>97\%$) was determined by SDS-PAGE, high-pressure liquid chromatography, and isoelectric focusing. 1E10 mAb was tested for sterility, pyrogenicity, mycoplasma, and viral contamination in accordance with the U.S. Food and Drug Administration guidelines (24). The immunogenic preparation was produced in accordance with the Good Manufacturing Practice guidelines and certified by the Quality Control Department of the Center of Molecular Immunology. Aluminum hydroxide (Superfos Biosector, Frederikssund, Denmark) was used as adjuvant. In brief, sterile purified 1E10 mAb was mixed at a final concentration of 2 mg/ml with 5 mg/ml aluminum hydroxide. The mixture was gently stirred for 3 h at room temperature. The aluminum hydroxide-precipitated mAb was aliquoted into pyrogen-free, sterile glass vials and stored at 4°C until use. These procedures were performed aseptically in a laminar flow hood. The final product was tested for sterility, pyrogenicity, and general safety in mice and guinea pigs before use according to United States Pharmacopeia (26).

mAb-biotin conjugation

mAbs were incubated with 100 $\mu\text{g/ml}$ *N*-hydroxysuccinimide biotin for 4 h at room temperature. Then, 20 μl of 1 M NH_4Cl per 250 μg of biotin were added and incubation was continued for 10 min to stop the reaction. Finally, mAb solutions were dialyzed extensively with PBS (25).

Preparation of F(ab')₂

mAb F(ab')₂ were obtained using a procedure previously described (26). mAbs were incubated in 2 mM citrate buffer, pH 3.5, in the presence of pepsin (Sigma-Aldrich) at 37°C, in an enzyme:mAb ratio of 1:20. The reaction was stopped after 4 h by raising the pH to 8 with 2 M Tris solution. The Fc fragments and the nondigested Ig were removed by absorption to protein A-Sepharose (Amersham Pharmacia Biotech), and the nonadsorbed fraction was eluted with 1.5 M glycine, 3 M NaCl buffer (pH 8.9). This fraction was extensively dialyzed with PBS and concentrated to at least 1 mg/ml. F(ab')₂ 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.

Selection of patients

Twenty patients with confirmed diagnosis of malignant melanoma in stage III and IV who had received standard therapy at least 4 wk before inclusion in the study and not susceptible to any other treatment were admitted to the study. Other eligibility criteria included a performance status according to World Health Organization degrees between 0–2, age from 18 to 80 years old of both sexes, life expectancy of 6 mo, and clinical laboratory parameters within normal limits. Patients were excluded for pregnancy or lactation, history of encephalopathy or convulsive syndrome, acute and severe allergic events, and acute infectious disease. Individual patient data are summarized in Table I. The trial protocol was approved by the Ethical Committee of the National Institute of Oncology and Radiobiology (Havana, Cuba), where the study was conducted, and by the National Regulatory Authority for Drug Quality Control. All patients signed informed consent forms after receiving complete information about the protocol in which they would be included. Before entering the study, all patients had a complete history and physical examination, complete blood cell count, chemistry profile, urinalysis, chest x-ray, evaluation of the performance

Table I. Characteristics of patients in study

Characteristics	No. of Patients
Patients entered into the study	20
Male/female	7/13
Age, years	
Median	57
Range	29–76
Site of disease	
Skin	10
Lymph node	7
Liver	3
Lung	3
Orbit	1
Maxillary sinus	1
Prior therapy	
Surgery	20
Radiation	2
Chemotherapy	2
IFN- α	4
Levamisole	4

status, and tumor measurements. Clinical and radiological evaluation were performed periodically.

Treatment schedule

Patients were treated with six doses of 2 mg of aluminum hydroxide-precipitated 1E10 mAb, injected intradermally into multiple sites at 14-day intervals. Serum was obtained pretreatment and 14 days after each immunization. Patients have been followed for a strict control of the possible adverse effects after each immunization.

Ab binding assays

The presence of Ab3 in sera obtained from melanoma patients was determined in a solid-phase ELISA. Wells of polystyrene Maxisorp microtiter plates (Nunc, Roskilde, Denmark; catalog no. 442404) were coated with 50 μl of a solution of 10 $\mu\text{g/ml}$ purified 1E10 mAb or its F(ab')₂ in carbonate buffer, pH 9.6, overnight at 4°C. Isotype-matched irrelevant mAbs were used as coating control Abs. After washing with PBS containing 0.05% Tween 20, the plates were blocked for 1 h at room temperature with PBS containing 1% BSA. Then, diluted serum samples were added to each well and the plates were incubated for 2 h at 37°C. After washing, alkaline phosphatase-conjugated secondary Abs were added. Alkaline phosphatase-conjugated goat anti-mouse IgG plus IgM (Jackson ImmunoResearch Laboratories, West Grove, PA; catalog no. 111-055-068), F(ab')₂ of goat anti-rabbit IgG (Sigma-Aldrich; catalog no. A-7778), and goat anti-human IgG plus IgM (Jackson ImmunoResearch Laboratories; catalog no. 109-055-127) were used. The plates were washed four times manually and the reaction was developed with a substrate solution consisting of 1 mg/ml *p*-nitrophenylphosphate (Sigma-Aldrich) in diethanolamine buffer, pH 9.8. Absorbance was measured at 405 nm in an ELISA reader (Organon Teknika, Salsburg, Austria). Assays were performed in triplicate for each sample and the SD was $<10\%$ for all values. Background values of absorbance corresponding to wells where no sera was added were <0.1 . Titer was defined as the highest serum dilution giving absorbance values ≥ 0.2 and being at least three times the value corresponding to the preimmune serum at the same dilution. Different murine mAbs were used in the study as controls: ior-CEA1 (IgG1, κ , anti-carcinoembryonic Ag), ior-C5 (IgG1, κ) against a glycoprotein expressed on human colorectal cells, and 14F7 (IgG1, κ) anti-NeuGc-GM₃ (27–29).

Binding of Ab3 to purified gangliosides was determined using an indirect ELISA as previously reported (30), with slight modifications. Briefly, gangliosides (200 ng/well) in 50 μl of methanol were dried in 96-well PolySorp Immunoplates (Nunc; catalog no. 475094). The wells were washed with PBS containing 0.2 M NaCl, 0.05% Tween 20 (PBS/Tween 20/NaCl), and the serum samples diluted in PBS/Tween 20 were incubated for 2 h at 37°C. After washing with PBS/Tween 20/NaCl, alkaline phosphatase-conjugated or biotinylated secondary Abs were added to the plates and incubated for 1 h at 37°C. In the case of biotinylated Abs, after washing in the same conditions, alkaline phosphatase-conjugated streptavidin was added and incubated for an additional hour at 37°C. Finally, a substrate solution consisting of 1 mg/ml *p*-nitrophenylphosphate in diethanolamine buffer, pH 9.8, was added to the plates. To consider that a serum sample had a positive reaction to a particular ganglioside, values of absorbance had

to be at least three times the absorbance value given by incubating the serum in wells containing no gangliosides.

The reactivity against standard gangliosides was also tested by enzyme immunostaining on high-performance thin layer chromatography (HPTLC) plates as previously reported (31).

For human Ig class analysis, alkaline phosphatase-conjugated goat anti-human IgG or anti-human IgM (Jackson ImmunoResearch Laboratories) were used as second Abs. The determination of human IgG subclasses was performed using biotinylated goat anti-human IgG1, IgG2, IgG3, or IgG4 (BD PharMingen, San Diego, CA; catalog nos. 35052D, 35072D, 35082D, and 35092D, respectively), followed by incubation with alkaline phosphatase conjugated to streptavidin (Jackson ImmunoResearch Laboratories) for 30 min at 37°C.

Ab binding inhibition assays

To ascertain whether Ab3 sera shared idiotopes with P3 mAb, sera were assessed for their ability to inhibit the binding of biotinylated 1E10 mAb to P3 mAb in an ELISA. In brief, a solution of 50 μ l of 1 mg/ml isotype-matched irrelevant mAb ior-c5 was added to microtubes containing 50 μ l of serial dilutions of immune sera diluted in PBS containing 1% BSA. After overnight incubation at 4°C, 100 μ l of biotinylated 1E10 mAb at nonsaturating concentration (7.8 ng/ml) were added to each sample and the mixtures were incubated at 37°C for 2 h. Then, 50 μ l of the samples were added to wells of Maxisorp microtiter plates previously coated with 10 μ g/ml P3 mAb (Ab1) and blocked with PBS 1% BSA. After a 1-h incubation at 37°C, plates were washed and bound 1E10 mAb was detected by adding streptavidin-alkaline phosphatase complex (Jackson ImmunoResearch Laboratories) for 30 min. The plates were washed and the reaction was developed with *p*-nitrophenylphosphate substrate (Sigma-Aldrich) in diethanolamine buffer, pH 9.8. Ab titer was considered as the highest dilution of sera, giving >20% of inhibition of the binding of 1E10 to P3 mAb.

The inhibition of the binding of P3 mAb to NeuGc-GM₃ by sera obtained from immunized patients was evaluated by ELISA. Microplate wells were coated with NeuGc-GM₃ and incubated with serial dilutions of pre- and postimmunization patients' sera. After washing with PBS, biotinylated-P3 mAb with 50% maximum reactivity against NeuGc-GM₃ (5 μ g/ml) was added to plates and incubated for 1 h at 37°C. Binding of P3 mAb to the ganglioside was detected by adding streptavidin-alkaline phosphatase complex (Jackson ImmunoResearch Laboratories) for 30 min. The plates were washed and the reaction was developed with *p*-nitrophenylphosphate substrate in diethanolamine buffer. Percentage of specific inhibition of P3 mAb binding was calculated relative to wells where no patient sera was added.

Evaluation of the anti-ganglioside reactivity after preabsorption of patients' sera with 1E10 mAb was performed by ELISA. Serum samples were preincubated with 1E10 mAb and added onto plates coated with NeuGc-GM₃. Serum reactivity was assessed by the ELISA procedures previously described. ELISA plates coated with 1E10 mAb were used as a control of the absorption efficiency.

Results

Twenty patients with advanced malignant melanoma, including 7 males and 13 females, have been enrolled in the study, with a mean age of 57 years (ranging from 29 to 76). At the moment of entry into the study, all patients had metastatic disease. Fourteen patients completed the entire vaccination schedule (six doses), five patients were removed from the study due to disease progression, and one patient abandoned the study. All patients had received different treatments previous to vaccination, listed in Table I.

Patients enrolled in the study were evaluated for safety. No evidence of unexpected or serious adverse effects was reported as a result of the 1E10 injection. Main toxicity included local reaction at the site of the injection with induration and local erythema, sometimes associated with mild pain, which resolved in a few days (1–3 days). Fever grade I-II (according to World Health Organization criteria), which resolved spontaneously or by usual antipyretic treatment, occurred in only a few patients. Chills and mild cephalgia occurred in few patients. The results obtained from routine hematology and hemochemistry laboratory tests included anemia grade I-II (World Health Organization) in six patients, increase of alkaline phosphatase grade I-II (World Health Organization) in two patients, and leukopenia grade I in two patients.

These and other adverse effects, such as increase of arterial tension, were interpreted by the physicians as definitely nonrelated to the product used. No other drug-related adverse effects were observed. The symptoms occurred independently of the number of doses administered to the patients, and this allowed classification of the toxicity as grade I (World Health Organization).

Although this study was not designed to evaluate therapeutic efficacy of the vaccine preparation, none of the patients has shown an objective clinical response to the 1E10 vaccine. However, the median time survival of the 14 patients who finished the treatment was 17 mo (95% confidence interval, 7.2–21.3), with seven patients with >1-year survival at the moment of the evaluation.

Patients who received at least four doses of the aluminum hydroxide-precipitated 1E10 mAb were considered as immunologically evaluable. The response induced by the immunogen was evaluated by analysis of patients' sera before and during the treatment. Sixteen of the 17 evaluable patients developed Abs against 1E10 mAb (human anti-mouse Abs). The reactivity of patients' sera with different isotype-matched mouse mAbs or their F(ab')₂ was compared by ELISA, and a preferential binding to the immunizing Ab (1E10) was observed. (Fig. 2).

The Ab response against 1E10 mAb was predominantly of the IgG isotype; no IgM Abs were detected at the lowest serum dilution tested (1/100). The titers of this Ab response ranged between 1/10,000 and 1/100,000 (Table II). Among the IgG subclasses, IgG1 and IgG4 were predominant in the Ab response against 1E10; a moderate presence of IgG2 and a minimal IgG3 response was also detected (data not shown).

In addition, Ab3 Abs expressing P3 idiotopes were detected in the hyperimmune sera from 16 of 17 studied patients as determined by the ability of patients' immune sera to inhibit 1E10 (Ab2) binding to P3 mAb (Ab1). The inhibition experiments were

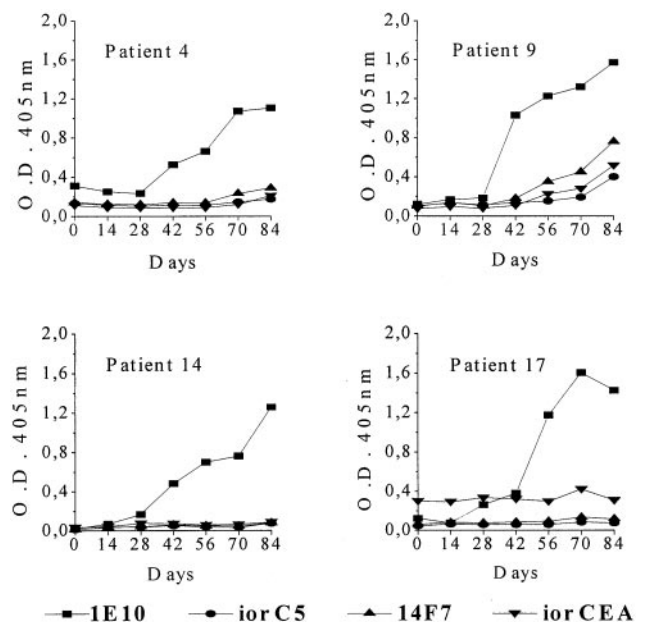


FIGURE 2. Specificity of Ab3 sera of vaccinated melanoma patients to 1E10 mAb (Ab2). Reactivity of sera from melanoma patients immunized with aluminum hydroxide-precipitated 1E10 mAb (IgG1) diluted 1/1000 was tested against F(ab')₂ (100 ng/well) of 1E10 and isotype-matched control Abs (ior-C5, 14F7, ior-CEA1) coated microtiter plates. Binding was assessed by using alkaline phosphatase-conjugated goat anti-human IgG plus IgM. Patients were immunized on days 0, 14, 28, 42, 56, and 70, and sera were obtained 14 days after each vaccination. The symbols represent the binding to different mAbs.

Table II. Ab3 response against 1E10 and NeuGc-GM₃ in vaccinated melanoma patients: maximal titer and isotype analyses

Patient	Anti-1E10		Anti-NeuGc-GM ₃	
	IgG	IgM	IgG	IgM
1	1/10,000		1/800	1/400
2	1/10,000			1/6,400
3	1/10,000			1/1,600
4	1/10,000		1/1,600	1/400
5	>1/100,000		1/1,600	1/6,400
6	1/10,000		1/3,200	1/6,400
7	1/10,000			1/6,400
9	1/10,000		1/3,200	1/12,800
11	1/10,000		1/400	1/6,400
13	1/10,000			1/400
14	1/50,000		1/3,200	1/3,200
15	1/10,000			1/1,600
16	1/100,000		1/800	
17	1/10,000		1/200	1/3,200
18	1/10,000		1/200	
20	1/10,000		1/800	1/800

performed by using patients' sera before vaccination and at the peak of the Ab response to 1E10 mAb. Sera obtained from patients were preabsorbed with an isotype-matched control mAb to block the anti-isotypic component of the Ab response, and the binding of biotinylated 1E10 to P3 mAb was evaluated. All sera from patients who developed Ab response against 1E10 mAb had the capacity to inhibit the binding of 1E10 to P3 mAb. Fig. 3 shows the percentage of inhibition for individual patients. It was found that at 1/2,000 serum dilution 14 of 16 patients showed >20% inhibition and even at sera dilutions of 1/10,000 seven patients still demonstrated >20% inhibition of 1E10 binding to P3 mAb. The specificity of the inhibition was confirmed, due to the inability of a hyperimmune serum obtained from a monkey immunized with another anti-Id mAb nonrelated to P3 mAb to inhibit the binding of 1E10 to P3 mAb. No inhibition was observed when preimmune sera from the patients were used.

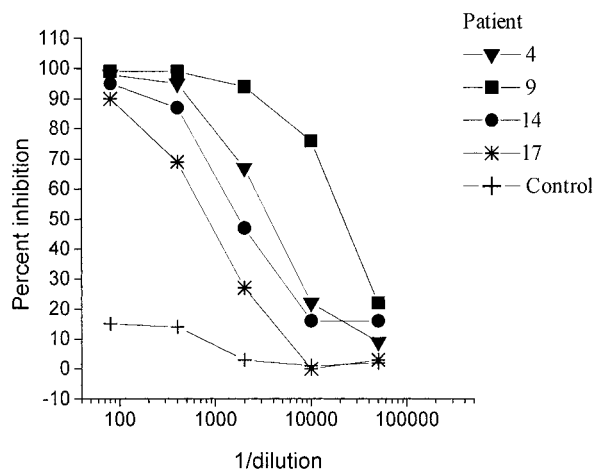


FIGURE 3. Ab3 sera of melanoma patients dosimetrically inhibit the binding of 1E10 mAb (Ab2) to P3 mAb (Ab1). Serial dilutions of Ab3 sera from vaccinated melanoma patients were mixed with 7.8 ng/ml biotinylated 1E10 mAb (Ab2) and added onto P3 mAb-coated microtiter ELISA plates (100 ng/well). A hyperimmune serum obtained from a monkey immunized with another anti-Id mAb nonrelated to P3 mAb was used as a control. Percentage of inhibition of biotinylated 1E10 was calculated relative to the binding in the absence of inhibitor.

To determine whether among the Ab3 Abs induced after vaccination with 1E10 there were Abs with the same Ag specificity as P3 mAb (Ab1), we tested by ELISA the binding of patients' sera with a panel of purified standard gangliosides including *N*-acetyl and *N*-glycolyl derivatives of different gangliosides. In contrast with the Ab response induced by 1E10 mAb in mice, specific Ab responses to NeuGc-GM₃ were found in all responder patients (16/17), and no reactivity with any of the *N*-acetylated gangliosides analyzed was observed (Fig. 4). Similar binding was detected when patients' sera were tested against NeuGc-GM₂. The Ab response against NeuGc-GM₃ ganglioside was increased with the course of vaccination, reaching a peak after patients received the fourth or fifth doses of the Ab2 mAb. Similar to anti-1E10 Ab responses, only sera from patient number 8 was not reactive with NeuGc-GM₃. Analysis of the isotype of the anti-NeuGc-GM₃ response indicated that 12 patients generated both IgG and IgM Abs, two patients showed only IgG, and in the other two patients only IgM Abs specific to NeuGc-GM₃ were observed. Ab titers of up to 1/12,800 (IgM) and 1/3,200 (IgG) were found, as summarized in Table II.

Evaluation of the IgG subclasses represented in the anti-NeuGc-GM₃ response showed the presence of Abs predominantly of IgG2 subclass, with low or no representation of other IgG subclasses in the majority of the patients. Inhibition of Ab1 P3 binding to the ganglioside NeuGc-GM₃ by patients' sera was also demonstrated. Preimmune serum from any patient had no inhibitory effect (Fig. 5).

Specificity of the anti-ganglioside Ab response was confirmed by HPTLC immunostaining by using sera from patients before treatment and at the peak of the Ab response. The results agreed with those obtained previously by ELISA and demonstrated a specific anti-NeuGc-GM₃/NeuGc-GM₂ serum reactivity in most patients studied. Fig. 6 shows results of the immunostaining with sera from patient 6, which specifically recognized the two *N*-glycolyl-containing gangliosides tested, as P3 mAb (Ab1) did (16, 17).

Due to the different isotype and subclass patterns of the Ab3 and the Ab1' (Ag-specific) responses induced after vaccination with 1E10, we addressed the question of whether a parallel cell subset producing natural Abs to NeuGc-containing gangliosides could be

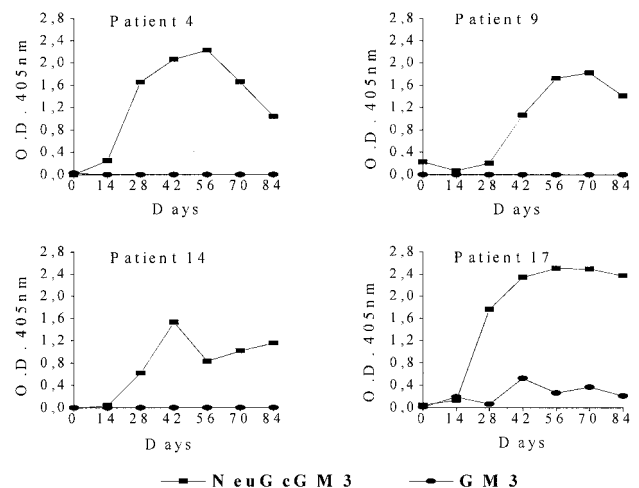


FIGURE 4. Specificity of Ab3 sera of vaccinated melanoma patients against NeuGc-GM₃. Reactivity of sera from melanoma patients immunized with aluminum hydroxide-precipitated 1E10 mAb (IgG1) diluted 1/400 was tested against NeuGc and NeuAc-GM₃ (100 ng/well). Binding was assessed by using alkaline phosphatase-conjugated goat anti-human IgG plus IgM. Patients were immunized on days 0, 14, 28, 42, 56, and 70, and sera were obtained 14 days after each vaccination.

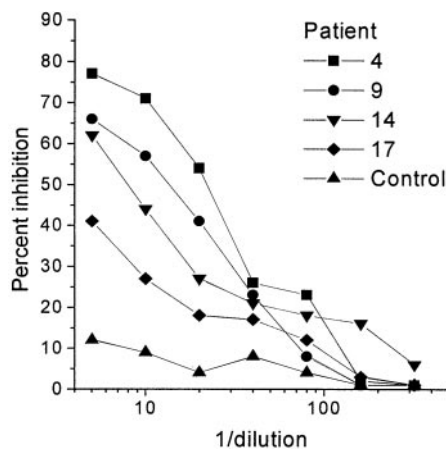


FIGURE 5. Inhibition of Ab1 P3 mAb binding to NeuGc-GM₃ ganglioside by patients' sera. Microtiter plates were coated with NeuGc-GM₃ and incubated with serial dilutions of patients' sera followed by incubation with biotinylated P3 mAb (Ab1). Preimmune serum from a patient was used as a control. Percentage of inhibition was calculated relative to binding in the absence of patients' sera.

expanded. We performed experiments where patients' sera were preincubated with 1E10 mAb and then added to ELISA plates coated with NeuGc-GM₃. There was statistical difference in the reactivity against the ganglioside between the nonadsorbed and adsorbed sera ($p < 0.05$, one sample t test), but this inhibition was not $>40\%$ of the original binding. Moreover, in some patients' sera the absorption with 1E10 mAb did not decrease the reactivity to NeuGc-GM₃. No reactivity of preadsorbed sera to 1E10-coated plates used as an absorption control was detected (Fig. 7).

A preliminary analysis of how long lasting was the Ab response against 1E10 mAb and NeuGc-GM₃ ganglioside showed that up to 4 mo after administration of the last dose with the vaccine preparation, specific Abs could be detected, representing 55–93% of the peak of the response.

Discussion

It is well established in several systems that monoclonal anti-Id Abs could potentially have a role in vaccine development by virtue of their ability to mimic tumor Ags and stimulate the immune system (32–37). Although Ab2 mAbs bearing the internal image of tumor-associated Ags are considered excellent candidates for immunotherapy, it has been demonstrated that the induction of Ag-specific humoral response due to the immunization with β -type Ab2 is not predictive of the biological effect induced by the Ab (38, 39).

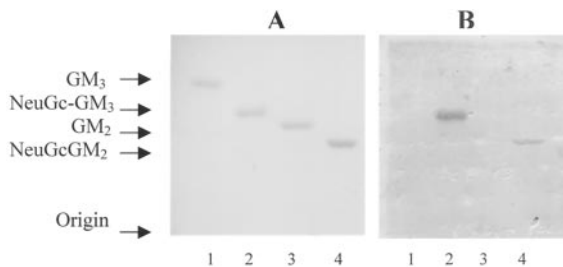


FIGURE 6. Specificity of Ab3 sera from patients for NeuGc gangliosides. HPTLC immunostaining of sera of an immunized patient. A, Control immunostaining of different gangliosides on HPTLC plates visualized with orcinol. Lane 1, NeuAc-GM₃; lane 2, NeuGc-GM₃; lane 3, NeuAc-GM₂; lane 4, NeuGc-GM₂. B, Binding of Ab3 sera (diluted 1/800).

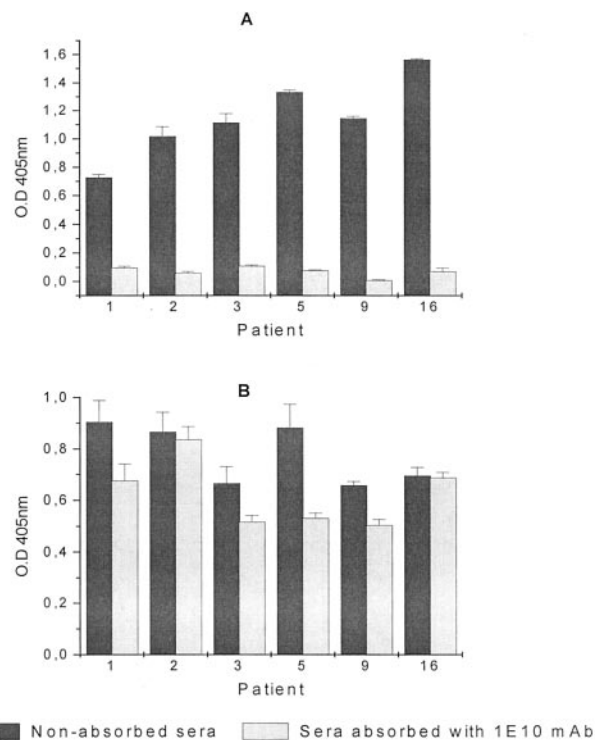


FIGURE 7. Ab3 reactivity against NeuGc-GM₃ in patients' sera absorbed with 1E10 mAb. Sera from melanoma patients were preincubated with 1E10 mAb and the remnant reactivity to 1E10 mAb (A) and NeuGc-GM₃ (B) was assessed by ELISA. Percentage of inhibition was calculated relative to the serum reactivity in the absence of preincubation with 1E10 mAb.

Preclinical studies performed in mice had shown that 1E10 was able to induce anti-anti-Id Abs (Ab3) that specifically bound to 1E10 and bear P3 mAb idiotopes, but it did not generate Ab3 Abs with the same Ag specificity as P3 mAb, suggesting that 1E10 mAb was not an internal image Ab2 and was initially classified as a γ -type anti-Id mAb (18, 19). Despite its "noninternal image" characteristics, 1E10 mAb was able to induce antitumor effects in murine tumor models (19).

According to these findings, and taking into consideration that the selection of an Ab2 mAb to be used for immunotherapy should be based on its biological effect more than its classification in α , β , or γ (40), we started a phase I clinical trial with a vaccine preparation containing the anti-Id mAb 1E10.

This study describes the results of a phase I clinical trial in patients with metastatic melanoma using the murine 1E10 anti-Id mAb. The study was conducted to assess the toxicity of the 1E10-aluminum hydroxide vaccine preparation and its capacity to induce immune responses in melanoma patients.

First, it is noteworthy that the treatment of the patients with repeated injections of this murine Ab2 mAb was well tolerated despite the development of anti-mouse Ig Abs. Although it is known that clinical trials using murine mAbs have demonstrated that repeated injections induce human anti-mouse Ig Abs in all patients, leading to the occurrence of potentially hazardous complications and lack of efficacy, our results have shown that the magnitude of the anti-isotypic response observed in most patients was lower than the corresponding anti-idiotypic response generated after vaccination with 1E10 mAb, a characteristic that had been previously found in preclinical experiments performed in monkeys immunized with the same vaccine preparation (data not shown). We found that 16 of the 17 patients that were considered

immunologically evaluable developed anti-mouse Ig Abs and all of them showed a preferential serological response to 1E10 mAb as compared with other isotype-matched mAbs used as controls, indicating the immunodominance of the idiotypic determinants of 1E10 mAb.

The induction of humoral immunity in patients treated with 1E10 mAb was clear not only because of its capacity to induce an Ab response against the murine Ig but also due to the demonstration of the presence of Ab3 Abs sharing P3 mAb (Ab1) idiotopes. The characteristic of generating Ab3 Abs that share idiotopes with P3 mAb (Ab1) has been a common feature observed in mice (18), rabbits, and monkeys immunized with 1E10 mAb (our unpublished observation). The capacity to induce this kind of Ab3 has also been reported by other groups using different anti-Id mAbs (41–44), indicating the capacity of anti-Id mAbs to generate “true” Ab3 Abs (Ab3, Id+).

Attempts to induce anti-ganglioside immune responses in melanoma patients have been conducted by other groups immunizing with anti-Id mAbs 1A7 and BEC2, mimicking disialogangliosides GD2 and GD3, respectively (7–9). Vaccination studies with BEC2 mAb have demonstrated the induction of anti-GD3 Ab response in a limited number of patients assessed by ELISA, and the results of clinical trials performed with 1A7 mAb have shown that all melanoma patients generated IgG Abs against GD2 ganglioside.

In contrast to previous results obtained in mice, rabbits, and monkeys, most patients in our study generated an Ab3 Ab response against NeuGc-GM₃ and NeuGc-GM₂, as demonstrated by direct binding to purified gangliosides assessed by ELISA, TLC immunostaining, and inhibition assays where sera obtained from immunized patients inhibited the binding of P3 mAb (Ab1) to NeuGc-GM₃, indicating that 1E10 mAb was able to induce the production of Ab3/Ab1' Abs (Ab3, Ag+). An explanation for the difference observed in the anti-ganglioside Ab response induced in patients as compared with mice, rabbits, and monkeys immunized with 1E10 anti-Id mAb could be due to the high expression of NeuGc-containing gangliosides in normal tissues of these animal species, being 1E10 vaccination unable to break the tolerance against these Ags. In contrast, there is a general agreement that the presence of NeuGc-containing gangliosides has not been demonstrated in normal human tissues (13), and the immunization with the anti-Id mAb 1E10 could then more easily induce an Ag-specific Ab response in melanoma patients. Although Ab responses to carbohydrate Ags such as gangliosides are generally of the IgM isotype, in several patients vaccinated with 1E10 mAb we detected a relatively high titer of anti-NeuGc-GM₃ Ab response not only of IgM but also of IgG isotype, suggesting a T cell cooperation in this response.

Previous attempts to characterize the specificity of the anti-ganglioside Ab3 Abs induced after vaccination with other anti-Id mAbs such as BEC2 showed the presence of anti-GD3 Abs in vaccinated patients' sera by ELISA, but they were unable to demonstrate Ag specificity against GD3 by immunostaining on TLC plates, probably because of low anti-GD3 Ab titers found in most patients (8). In contrast, we have been able to show a specific reactivity of the Ab3 Abs to NeuGc-GM₃ and NeuGc-GM₂ not only by ELISA but also by TLC immunostaining. To our knowledge, this is the first report describing the generation of a specific immune response to tumor-associated NeuGc gangliosides induced after vaccination with an anti-Id mAb that can be also demonstrated by TLC immunostaining. The recognition of other NeuGc glycoconjugates different from gangliosides in human tumors by Abs present in patients' sera has to be demonstrated in the near future.

It is important to note the fact that a different Ig isotype pattern was produced against 1E10 Ab molecule in comparison with the one generated against the gangliosides. This finding suggests that two different populations of B cells could recognize the gangliosides. Results from inhibition experiments showed that when serum reactivity against 1E10 mAb is abrogated by adsorbing the patient sera with this Ab2 Ab, remaining reactivity against *N*-glycolyl gangliosides is measurable, suggesting the presence of nonclassical Ab1' (Id-Ag+) Abs. Naturally occurring Abs in response to NeuGc-containing gangliosides in tumor-bearing patients could contribute to this unusual Ab parallel subset. Such nonclassical Ab1' (Id-Ag+) Abs might recognize nonparatopic idiotopes into Ab3 (Id+) Abs. In summary, 1E10 mAb vaccination can most likely induce anti-NeuGc-containing ganglioside Abs not only because of a classical Ag mimicry effect but also by activating naturally occurring idiotypic networks. We have experimental data in syngeneic mice supporting the idea of a B-T cell idiotypic network associated to P3 and 1E10 mAbs.⁴ We cannot exclude at present the involvement of T cells in the immunomodulatory effect of 1E10 vaccination.

The immune response generated in the patients and the low rate of side effects observed justify the development of new clinical trials in cancer and in patients with minimal residual disease in an adjuvant approach after conventional treatments and the continuation of the study of the mechanisms through which the vaccine can exert a biological effect in patients. Ongoing clinical trials by using 1E10 vaccine include studies in patients with advanced breast cancer and small cell lung cancer.

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