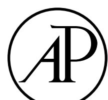
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Immune responses in breast cancer patients immunized with an anti-idiotype antibody mimicking NeuGc-containing gangliosides

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Abstract

A phase I clinical trial was conducted in patients with stage III/IV breast cancer who were treated with the anti-idiotype mAb 1E10 specific to an Ab1 mAb able to react specifically with *N*-glycolyl-containing gangliosides and with antigens expressed on human melanoma and breast carcinoma cells. Patients were treated with 1 or 2 mg of aluminum hydroxide-precipitated 1E10 mAb every other week for six injections. Two patients at each dose were reimmunized 7–9 months after completing the induction phase. In hyperimmune sera from eight of the nine patients who received at least four doses of anti-Id vaccine preparations, strong specific responses were observed both against 1E10 mAb and NeuGc-GM_3 ganglioside (Ab3 Id^+Ag^+). Nonclassical Ab1′ antibodies (Id^-Ag^+) were also elicited by 1E10 mAb vaccine treatment. There were no differences between the two levels of dose tested in relation to toxicity and immunogenicity. No evidence of serious or unexpected effects was observed.
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Keywords: Anti-idiotypic antibodies; Gangliosides; Clinical studies; Immunotherapy; Cancer vaccines

Introduction

Gangliosides are surface membrane glycosphingolipids containing sialic acid residues expressed on the cell surface membranes of most mammalian cell types [1]. These glycolipids are considered attractive targets for cancer immunotherapy due to the changes that occur in their expression pattern during malignant transformation, but their carbohydrate and self-nature make them poorly immunogenic. Different strategies to increase the immune response of cancer patients against gangliosides include the coupling of these antigens to a carrier protein and their administration combined with potent adjuvants [2,3].

An alternative approach to generating improved immune responses to these molecules is the use of anti-idiotype (Ab2) antibodies bearing the "internal image" of the antigen (Fig. 1). Ab2 mAbs able to mimic gangliosides have induced humoral responses against the nominal ganglioside and have generated antitumor responses in animal models [4–7]. In the clinical setting, promising results have been reached using Ab2 mAbs as ganglioside surrogates to treat cancer patients [8–13], but no immunochemical surrogate of patient clinical outcomes has yet been found.

The presence of gangliosides bearing *N*-glycolyl (NeuGc)-neuraminic acid residues in normal human tissues has not been demonstrated, but in contrast the expression of these types of gangliosides in several human tumors has been reported [14–17], which makes these antigens attractive candidates for cancer immunotherapy.

Our group has previously described an Ab2 mAb to a murine Ab1 mAb, named P3, which selectively binds NeuGc-neuraminic acid on several monosialo- and disialogangliosides [18,19] and also reacts with antigens expressed in human melanomas and breast tumors [18,20]. This IgG1 Ab2 mAb, designated 1E10, was able to inhibit the binding

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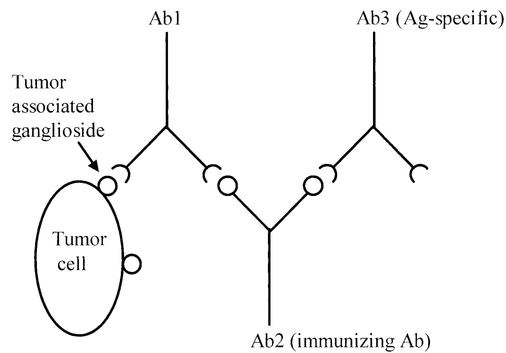

Fig. 1. Relationship between the antibodies in the idiotypic cascade.

of P3 mAb to NeuGc-GM_3 ganglioside and to induce in syngeneic animals anti-anti-idiotypic antibodies (Ab3) bearing P3 mAb idiotopes, but it was unable to generate Ab3 antibodies with the same antigen specificity as P3 mAb [21]. This "noninternal image" Ab2 was capable of inducing a strong antitumor effect in tumor-bearing mice [22]. In a phase I clinical trial performed in advanced melanoma patients immunized with 1E10 mAb, the treatment proved to be safe and immunologically effective. In addition to the development of Ab3 antibodies capable of inhibiting Ab2 binding to Ab1 (Ab3Id^+), a specific Ab3 response to NeuGc-containing gangliosides was also elicited in patients due to 1E10 mAb immunization. These results showed an "internal image" behavior for 1E10 Ab2 mAb in humans, in contrast with our previous results obtained in mice, rabbit, and monkeys. Strikingly, Ab1′ antibodies able to bind to NeuGc-containing gangliosides, but not to 1E10 mAb (Id-Ag^+), were detected in immunized patients' sera [20].

In this paper we report the results of a phase I clinical trial in which advanced breast cancer patients were treated with two different dose levels of 1E10 mAb, in a treatment schedule consisting of an immune response induction phase followed by a reimmunization phase. Our data enlarge the results obtained previously in melanoma patients treated with this Ab2 vaccine and additionally provide evidence that repeated applications of 1E10 mAb are safe and useful for maintaining anti-anti-Id and antigen-specific antibody responses in patients.

Materials and methods

Gangliosides

Gangliosides, NeuAc-GM_3, NeuGc-GM_3, and NeuGc-GM_2 were kindly provided by Dr. L.E. Fernández, Vaccine Department, Center of Molecular Immunology (Havana, Cuba). NeuAc-GM_3 and NeuGc-GM_3 gangliosides were purified from dog and horse erythrocytes, respectively, as described [23]. NeuGc-GM_2 was purified from mouse liver [24]. GM_2 from bovine brain was purchased from Sigma (St. Louis, MO). The homogeneity and purity of gangliosides was more than 95% as determined by thin-layer chromatography and densitometry [25].

Preparation of 1E10 mAb for the clinical trial

1E10 Ab2 mAb (IgG1,κ) was generated by immunizing BALB/c mice with P3 mAb (IgM, κ) [18] as previously described [21]. 1E10 mAb was purified from mouse ascites in the Good Manufacturing Practice (GMP) 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 4B columns and size exclusion chromatography on Sephadex G-25 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated immunoglobulins was more than 97% as 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 Food and Drug Administration guidelines [26]. The vaccine was produced in accordance with the Good Manufacturing Practice guidelines and certified by the Quality Control Department of the Center of Molecular Immunology. Briefly, sterile purified 1E10 mAb was aseptically mixed at a final concentration of 1 or 2 mg/ml with 5 mg/ml of aluminum hydroxide as adjuvant (Superfos Biosector, Frederikssund, Denmark). 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. The final product was tested for sterility, pyrogenicity, and general safety in mice and guinea pigs before use according to the United States Pharmacopeia [27].

Conjugation of mAbs to biotin

This procedure was performed as described [28]. mAbs at a concentration of 1 mg/ml were incubated with 100 μg/ml of *N*-hydroxysuccinimide biotin in DMSO for 4 h at room temperature. The reaction was stopped by adding 20 μl of 1 M NH_4Cl per 250 μg of biotin to the reaction mix. Finally, mAb solutions were dialyzed extensively with PBS.

Preparation of $F(ab')_2$ fragments

The mAb $F(ab')_2$ fragments were obtained using a standard procedure previously described [29].

Selection of patients

Ten female patients with confirmed diagnoses of breast cancer in stage III or IV or metastatic diseases were selected for the study. Other inclusion criteria were a performance status according to WHO degrees between 0 and 2, age from 18 to 80 years old, minimal life expectancy of 6 months, and

clinical laboratory parameters within normal values. Patients had received the last standard therapy at least 4 weeks before inclusion in the study. The main exclusion criteria for patients were pregnancy or lactation, brain metastases or previous history of encephalopathy, acute and severe allergic events, chronic or acute infectious disease, and autoimmune diseases. The trial protocol was approved by the Ethical Committee of the Center of Medical Surgical Research (Havana, Cuba), where the study was developed, and authorized by the National Regulatory Authority for Drug Quality Control (CECMED). All patients signed informed consent forms after receiving complete information about the protocol in which they would be included. Prior to enter onto 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 status, and tumor measurements. Clinical and radiological evaluations were performed periodically.

Treatment schedule

Patients were treated intradermally with either 1 or 2 mg of aluminum hydroxide-precipitated 1E10 mAb every other week for six injections (immune response induction phase). Serum samples were obtained before treatment and 14 days after each immunization. Four patients, two from each dose level, were reimmunized 7–9 months after the end of the induction phase and have continued receiving 1E10 mAb injections every 3 months to the present time. Patients have been followed for possible adverse effects after each dose of the vaccine.

Antibody binding assays

To measure Ab3 reactivity in sera from breast cancer patients, a solid-phase ELISA was performed as previously described [20], using microtiter MaxiSorp plates (Nunc, Roskilde, Denmark, Catalog No. 442404) coated with 500 ng/well of purified 1E10 mAb or its $F(ab')_2$ fragments. As isotype-matched controls used were ior cea-1 (anti-carcinoembrionic antigen), ior C5 (against a glycoprotein expressed on human colorectal cells), and 14F7 (anti-NeuGc-GM_3) mAbs and their $F(ab')_2$ fragments [30–32]. Absorbance was measured at 405 nm in an ELISA reader (Organon Teknika, Salsburg, Austria). All samples were assayed in triplicate and the standard deviation was less than 10% for all values. The highest serum dilution giving optical density values ≥ 0.2 and being at least three times the value corresponding to the preimmune serum at the same dilution was considered as titer. Background values in the absence of sera were less than 0.1.

Reactivity of Ab3 to gangliosides was assessed by an ELISA as previously reported [20]. Biotinylated secondary antibodies were used and reaction was developed as described. A serum sample was considered to bind to a particular ganglioside when absorbance values were at least three times the value obtained by incubating the serum in wells containing no gangliosides.

Enzyme immunostaining on HPTLC plates was also performed to determine the reactivity of sera against standard gangliosides, as previously described [33].

The pattern of IgG subclasses developed in patients due to the vaccination was determined using biotinylated goat antihuman IgG1, IgG2, IgG3, or IgG4 (Pharmingen, Catalog Nos. 35052D, 35072D, 35082D, and 35092D), as previously reported [20].

Inhibition assays

To define the extent of the idiotype-specific response against 1E10 mAb, patient's hyperimmune sera were incubated overnight at 4°C with 1 mg/ml of isotype-matched irrelevant mAb ior cea-1 to absorb the human antibodies against the isotypic determinants of 1E10 mAb. Then, samples were added onto 1E10 mAb-coated MaxiSorp plates for 2 h at 37°C. After washing with PBS–0.05% Tween 20, the plates were incubated with alkaline phosphatase-conjugated goat antihuman IgG + IgM (Jackson Immunoresearch Laboratories) for 1 h at 37°C. The reaction was developed by adding 1 mg/ml of *p*-nitrophenylphosphate in diethanolamine buffer, pH 9.8; ior cea-1-coated plates were used to measure the isotypic response in normal sera and as controls of the absorption efficiency.

Also, sera from patients were assayed by ELISA for their ability to inhibit the binding of 1E10 mAb to P3 mAb, as previously described [20]. Isotype-matched mAb ior cea-1 was used to absorb the anti-isotypic antibody fraction of the sera responses against 1E10 mAb. The percentage of inhibition of 1E10 mAb binding to P3 mAb was calculated relative to the binding of biotinylated 1E10 mAb in the absence of inhibitor. The antibody titer was defined as the highest serum dilution giving more than 20% of inhibition. To assess whether patients' sera were capable of inhibiting binding of Ab1 P3 mAb to NeuGc-GM_3 ganglioside, an ELISA was performed as previously reported [20], in which serial dilutions of preimmune and hyperimmune sera in PBS–BSA were mixed with 80 ng/ml of biotinylated P3 mAb (50% maximal reactivity to NeuGc-GM_3) and added onto plates coated with NeuGc-GM_3. The percentage of inhibition was calculated in relation to the binding of P3 mAb to NeuGc-GM_3-coated wells in the absence of patients' sera.

Reactivity of patients' sera to NeuGc-GM_3 after absorption with 1E10 mAb was evaluated by an ELISA as previously described [20]. Binding of preabsorbed sera to 1E10-coated plates was used as control of absorption efficiency. The percentage of inhibition was calculated in relation to the reactivity of sera to NeuGc-GM_3 in the absence of 1E10 mAb.

Purification of 1E10 mAb-specific antibodies

Hyperimmune sera from a breast cancer patient were pooled and passed over an immunoadsorbent column con-

Table 1
Characteristics of patients included in the study

Patient No.	Age	Disease stage	Prior therapy	1E10 mAb treatment	
				Dose level (mg)	No. of doses
01	62	Metastatic disease	Surgery, chemotherapy, radiotherapy, hormone therapy	1	6
02	62	IIIb	Hormone therapy	1	10
03	68	Metastatic disease	Surgery, radiotherapy, hormone therapy	1	9
04	56	Metastatic disease	Surgery, chemotherapy, radiotherapy, hormone therapy	1	6
05	38	Metastatic disease	Surgery, chemotherapy, radiotherapy, hormone therapy	1	2
06	72	Metastatic disease	Surgery, chemotherapy, radiotherapy, hormone therapy	2	10
07	63	Metastatic disease	Surgery, chemotherapy, radiotherapy, hormone therapy	2	5
08	60	Metastatic disease	Surgery, radiotherapy, hormone therapy	2	8
09	41	IV	Chemotherapy, hormone therapy	2	5
10	62	Metastatic disease	Surgery, chemotherapy, radiotherapy	2	6

sisting of immunizing 1E10 mAb coupled to Sepharose 4B. Protein bound to the column was eluted with glycine–HCl (pH 2.8) and neutralized with 2 M Tris. The reactivity of the eluted and unbound fractions against 1E10 mAb and gangliosides was tested by ELISA as described.

Results

Toxicity and clinical outcome

Ten patients with advanced breast cancer, all female, were recruited in the study (Table 1). The median age was 58.5 years, ranging from 38 to 72. Eight patients had metastatic disease at the moment of the inclusion, with metastases in different organs, including bones, lungs, lymph nodes, and subcutaneous cell tissue; one patient was in stage IIIb and one patient was in stage IV. Seven patients received six doses of 1E10 mAb vaccine preparation, thus completing the immune response induction phase. Among them, four patients were reimmunized, receiving a total number of 8 to 10 injections of anti-Id vaccine. Two patients were removed from the trial after receiving five doses due to disease progression, and one patient abandoned the study after receiving two doses of the vaccine. All patients had been treated with standard therapies before entering the study, including surgery, radiotherapy, chemotherapy, and hormone therapy.

Toxicity of the vaccination with 1 or 2 mg of aluminum hydroxide-precipitated 1E10 mAb was classified as grade I (according to World Health Organization criteria). Severe or unexpected adverse effects were not observed during the treatment or the follow-up period. The main symptoms of toxicity included local erythema and induration, in some cases accompanied with mild pain which resolved in a few days (1–3 days). Other reactions observed in a few patients were chills, cephalea, and fever grade I-II (WHO) that resolved spontaneously or by antipyretics. These symptoms were independent of the number of injections administered to the patients or the dose (1 or 2 mg). The increase in blood pressure that occurred in three patients was felt to be unrelated to the product used, as judged by the treating physicians. No other drug-related adverse effects were observed.

Clinical responses were monitored although this study was not designed to evaluate the therapeutic efficacy of the vaccine preparation. The median overall time to progression was 8 months (95% confidence interval, 7.08–8.92 months). In the group of patients who received up to six doses of the vaccine preparations the median time to progression was 7 months. In contrast, the median time to progression for the four patients who received more than six doses of aluminum hydroxide-precipitated 1E10 mAb was 15 months; two of these reimmunized patients remained with stable disease for 27+ and 25+ months, respectively. The median survival time of all treated patients was 19 months. In the case of the five patients treated with five to six doses of the anti-Id mAb the median survival time was 7 months (95% confidence interval, 2.71–11.29 months) and for those that received more than six doses was 24 months (95% confidence interval, 20.61–27.39 months).

Immunological responses

Nine patients who received at least four doses of aluminum hydroxide-precipitated 1E10 mAb were considered immunologically evaluable. Antibody responses induced by immunization with 1E10 mAb were tested in sera obtained from patients before and during the treatment. Eight patients developed antibodies against the whole molecule of 1E10 mAb (human antimouse antibodies) and its $F(ab')_2$ fragments during the induction phase. The maximum Ab3 responses corresponding to this phase were found 14 days after receiving the last dose of the vaccine preparation. The kinetics of Ab3 responses induced in four representative patients are shown in Fig. 2.

To confirm that a specific response against 1E10 mAb idiotype was generated by the immunization, patients' hyperimmune sera were preabsorbed with an irrelevant isotype-matched mAb, and the remaining reactivity against 1E10 mAb was measured by ELISA. As shown in Fig. 3, a

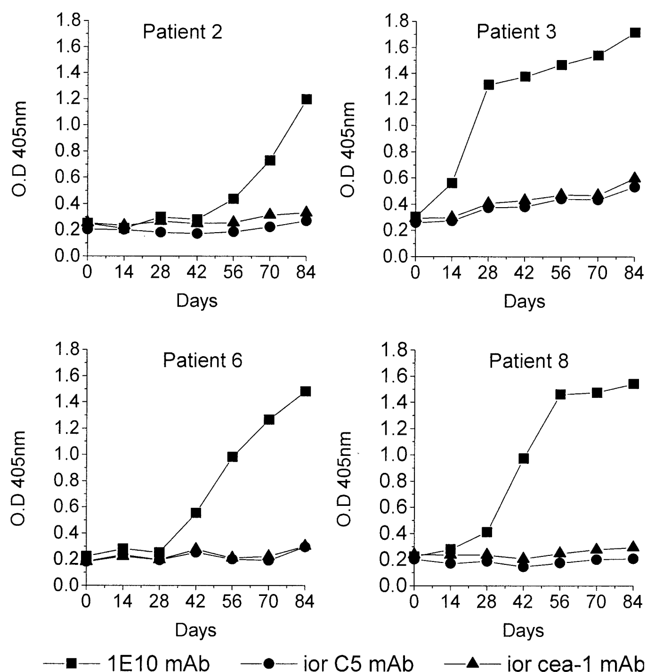

Fig. 2. Specificity of Ab3 antibodies in sera from breast cancer patients to 1E10 mAb assessed by ELISA. Sera from vaccinated patients diluted 1/1000 were bound to microtiter plates coated with 1E10 mAb and isotype-matched control mAb F(ab′)$_2$ fragments (500 ng/well), and the reaction was developed with alkaline phosphatase-conjugated goat antihuman IgG + IgM. Injections were given on days 0, 14, 28, 42, 56, and 70, and sera were obtained 14 days after each vaccination.

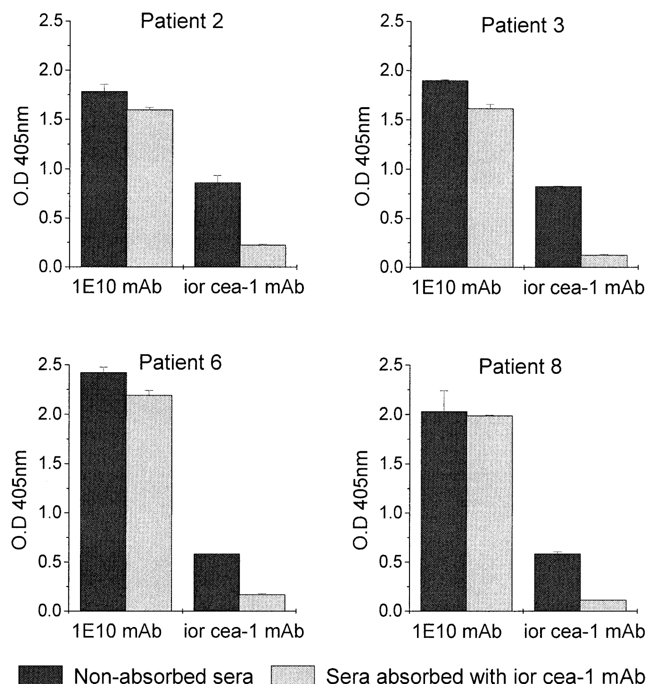

Fig. 3. Immunodominance of 1E10 mAb idiotype in the antibody response induced in vaccinated breast cancer patients. Hyperimmune sera from patients immunized with aluminum hydroxide-precipitated 1E10 mAb were preincubated with the isotype-matched ior cea-1 mAb, and later the reactivity against 1E10 and ior cea-1 mAbs was assessed by ELISA.

strong reactivity against 1E10 mAb was detected in patients' sera after preabsorption with the control mAb. The level of antibody response against the 1E10 mAb idiotype was higher than that against the isotype response in all patients ($P < 0.05$, Mann–Whitney test).

The titers of Ab3 response in the induction phase ranged from 1:10,000 to more than 1:100,000 as measured by ELISA (Table 2). This response was mostly of IgG isotype; no IgM antibodies were detected at the lowest serum dilution tested (1:100). The analysis of IgG subclasses showed a predominance of IgG1- and IgG4-type antibodies (data not shown).

The presence of P3 mAb idiotopes in Ab3 antibodies was proved due to the ability of hyperimmune sera from all responder patients to inhibit specifically the recognition of P3 mAb (Ab1) by biotinylated 1E10 mAb (Ab2), as shown in Fig. 4. No inhibition was observed when hyperimmune serum from a monkey immunized with an irrelevant anti-idiotypic mAb or preimmune sera from patients were used as control for specificity. It is noteworthy that even at sera dilutions of 1:250,000, three patients showed more than 20% specific inhibition.

To determine if Ab3 antibodies with the same specificity as P3 mAb could be elicited in breast cancer patients after vaccination with 1E10 mAb, sera obtained before and during the immunization protocol were tested by ELISA for the recognition of several *N*-acetyl and *N*-glycolyl derivatives of different purified standard gangliosides. All patients who developed Ab3 responses were also able to generate IgM and IgG antibodies that specifically bound to *N*-glycolyl variants of the gangliosides tested. Titers of up to 1:12,800 and 1:6400 of IgM and IgG responses were obtained, respectively (Table 3). No reactivity was observed with any of the gangliosides having NeuAc as their sialic acid residue. This antibody response against *N*-glycolyl-containing gangliosides reached a peak after patients received four or five doses of 1E10 mAb (Fig. 5). It was noteworthy that two patients who mainly developed an early and strong IgM antibody response against these gangliosides (patients 2 and

Table 2
Titers and isotype of Ab3 response against 1E10 mAb in vaccinated breast cancer patients

Patient	1E10 mAb	
	IgG	IgM
1	1/10000	—
2	1/50000	—
3	>1/100000	—
4	1/100000	—
6	>1/100000	—
7	>1/100000	—
8	>1/100000	—
9	—	—
10	1/10000	—

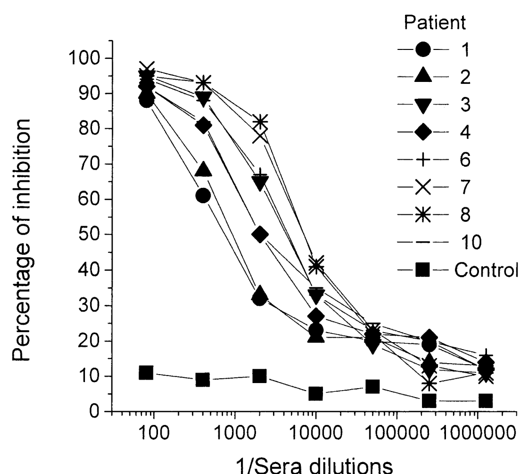

Fig. 4. Specific inhibition of binding of 1E10 mAb (Ab2) to P3 mAb (Ab1) by immune sera from breast cancer patients. Binding of biotinylated 1E10 mAb to P3 mAb-coated plates was tested in the presence of serial dilutions of patients' sera. A hyperimmune serum from a monkey immunized with a nonrelated anti-Id mAb was used as control. The percentage of inhibition was relative to the binding of biotinylated 1E10 mAb to P3 mAb in the absence of serum.

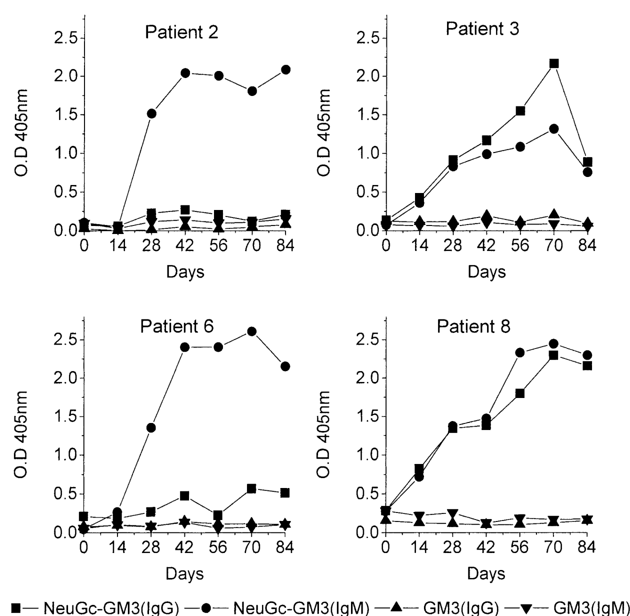

Fig. 5. Kinetics and specificity of IgG and IgM responses against NeuGc-GM_3 in sera from breast cancer patients assessed by ELISA. Sera from vaccinated patients diluted 1/400 were bound to microtiter plates coated with NeuGc and NeuAc-GM_3 (200 ng/well), and the reaction was developed with biotinylated goat antihuman IgG and antihuman IgM, followed by the addition of an alkaline phosphatase–streptavidin complex. Injections were given on days 0, 14, 28, 42, 56, and 70, and sera were obtained 14 days after each vaccination.

6, Fig. 5 and Table 3) showed quite different kinetics of Ab3 response against the 1E10 mAb molecule (Fig. 2). These two patients were those who remained with stable disease for 27+ and 25+ months.

The analysis of the distribution of IgG subclasses revealed in most patients (7) the presence of IgG2 antibodies against NeuGc-GM_3; IgG1 antibodies were detected in some patients (3) and very low or no representation of other IgG subclasses was found (data not shown).

The specificity of this response was also confirmed by immunostaining in HPTLC plates, where an evident specific binding of hyperimmune patients' sera with the NeuGc-containing gangliosides tested was observed. In all cases stronger serum reactivity was observed with NeuGc-GM_3 than with NeuGc-GM_2 (Fig. 6). No reaction was observed when preimmune sera from patients were used.

A further demonstration of the generation of true Ab1′ antibodies in patients by 1E10 mAb immunization was the ability of patients' hyperimmune sera to inhibit the binding of P3 mAb to NeuGc-GM_3 ganglioside. No inhibition was observed when patients' preimmune sera were used (Fig. 7).

Table 3
Titers and isotype of antibody response against NeuGc-GM_3 in vaccinated breast cancer patients

Patient	NeuGc-GM_3	
	IgG	IgM
1	1/800	1/3200
2	1/400	1/6400
3	1/3200	1/400
4	1/3200	1/6400
6	1/800	1/12800
7	1/3200	1/12800
8	1/6400	1/12800
9	—	—
10	1/400	1/1600

The kinetics of the anti-NeuGc-GM_3 antibody response in the four patients who were reimmunized with 1E10 mAb was studied. Antibodies against the ganglioside were still present in all patients 7 to 9 months after completing the induction phase of the treatment. Titers up to 1/800 for both IgG and IgM were detected just before starting the reimmunization phase, indicating the generation of a long-lasting antibody response due to the induction schedule. Subsequent reimmunizations with the anti-Id mAb resulted in an increase in the anti-NeuGc-GM_3 antibody titers. Although the maximum response of the induction phase was never reached, the antibody titers were sustained over 1/1600 during the last months of the treatment. Fig. 8 shows the data obtained from two representative patients.

We also tested the ability of patients' sera to bind to NeuGc-GM_3 after preincubation with 1E10 mAb. As Fig. 9A shows, reactivity against the ganglioside in preabsorbed sera diminished in comparison with that obtained with non-absorbed sera ($P < 0.05$, Mann–Whitney test), indicating that the immunization of the patients with 1E10 mAb induced Ag^+Id^+ antibodies. However, a significant binding to NeuGc-GM_3 was still detected in preabsorbed sera of all patients, suggesting the presence of a fraction of antibodies characterized to be Ag^+Id^-. Plates coated with 1E10 mAb

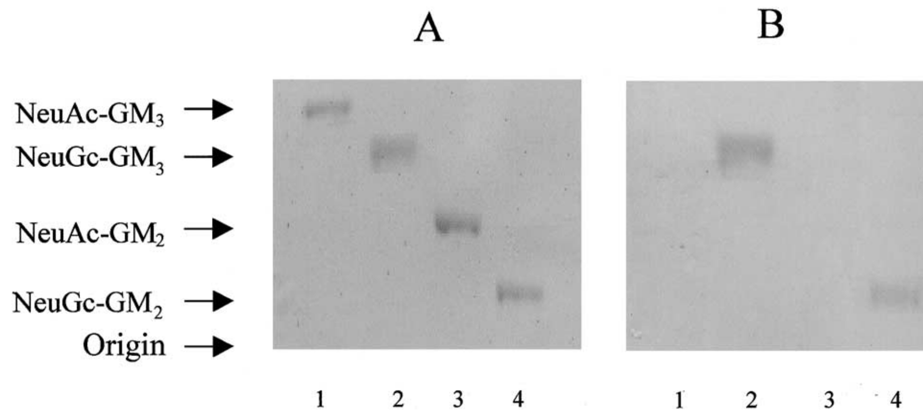

Fig. 6. Specific reactivity of sera from breast cancer patients to NeuGc gangliosides assessed by HPTLC immunostaining. (A) Gangliosides were chromatographed with chloroform:methanol:0.2% $CaCl_2$ in 2.5 M NH_3 (5:4:1, vvv) and visualized with orcinol. Lane 1, NeuAc-GM_3; lane 2, NeuGc-GM_3; lane 3, NeuAc-GM_2; lane 4, NeuGc-GM_2. (B) Binding of immune sera from patient 7, diluted 1/100.

were used as controls of the absorption efficiency, and the binding to the Ab2 was completely abrogated in preabsorbed sera (Fig. 9B). This result was confirmed when 1E10 mAb-specific antibodies were purified from a hyperimmune patient's serum by immunoadsorption in a Sepharose-4B-coupled 1E10 mAb column. Both the bound and the unbound antibody fractions showed specific reactivity against NeuGc-GM_3 ganglioside. No reactivity against 1E10 mAb could be detected in the unbound fraction (data not shown).

Discussion

In this study we report the results of a phase I clinical trial performed in advanced breast cancer patients with 1E10 mAb vaccine. Our primary goals were to evaluate

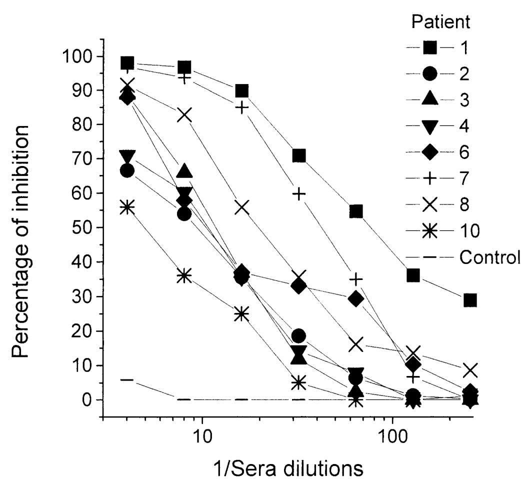

Fig. 7. Inhibition of binding of P3 mAb to NeuGc-GM_3 ganglioside by immune sera from breast cancer patients. Serial dilutions of patients' sera were added onto NeuGc-GM_3-coated microtiter plates, followed by incubation with biotinylated P3 mAb. Preimmune serum from a patient was used as control of the experiment. The percentage of inhibition was calculated relative to reactivity in the absence of sera.

toxicity and immune responses generated by the vaccine preparation in these patients and to compare the effects of two different doses of the anti-Id mAb.

Eight of the nine patients regarded as immunologically evaluable developed a high titer of IgG antibody response against 1E10 mAb. There were no differences in the responses generated in patients treated with 1 or 2 mg of aluminum hydroxide-precipitated 1E10 mAb. The higher binding of immunized patients' sera with 1E10 mAb in comparison with reactivity against isotype-matched control antibodies suggested the immunodominance of the 1E10 mAb idiotype in the antibody response. This finding was reinforced by the results of experiments where patients' sera were incubated with an irrelevant IgG1 mAb to absorb the reactivity against the isotype of 1E10 mAb. An evident reactivity against the immunizing antibody was detected in preabsorbed sera, indicating a strong recognition of the 1E10 mAb idiotype. In addition, the magnitude of the anti-idiotypic response was significantly higher than the response detected in nonabsorbed sera against the control antibody (anti-isotypic response). The immunodominance of the idiotypic determinants of 1E10 mAb has been reported in a previous phase I clinical trial carried on by our group in advanced melanoma patients treated with 1E10 mAb [20]. Although this predominant immunogenicity of an idiotype has been also demonstrated in other anti-Id mAbs [34], in our experience it is not a general property of this kind of antibody. When monkeys were immunized by us with another IgG1 anti-Id mAb against a mAb specific to NeuAc-GM2 [35], the antibody response against the isotype was predominant (our unpublished observation).

It is noteworthy that, in spite of the repeated injections of the 1E10 mAb vaccine and the strong responses generated to the murine immunoglobulin, the treatment with this antibody was well tolerated, confirming the safety of this anti-Id vaccine preparation reported in the previous trial in advanced melanoma patients [20].

The anti-anti-idiotypic antibodies elicited by 1E10 mAb

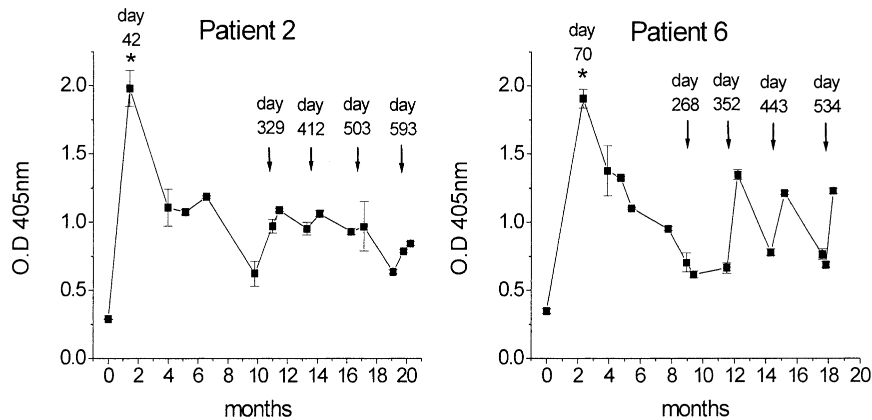

Fig. 8. Kinetics of anti-NeuGc-GM_3 antibody responses in patients reimmunized with aluminum hydroxide-precipitated 1E10 mAb. Serum reactivity with NeuGc-GM_3 was measured as described under Materials and Methods. The asterisk indicates the peak of the response against NeuGc-GM_3 obtained in the induction phase. The arrows indicate the moments when the patients were boosted with the Ab2 mAb.

in breast cancer patients were true Ab3 antibodies, as evidenced by the strong inhibition of Ab2 binding to Ab1 (P3 mAb) by patients' immune sera, as this indicates the presence of antibodies sharing idiotopes with P3 mAb.

The results of clinical trials using the anti-Id 1A7 mAb to immunize melanoma patients have demonstrated the capacity of this mAb to induce specific IgG anti-GD2 responses in patients that were maintained after monthly reimmunizations [10,13]. Also, specific anti-GD3 antibody responses were elicited in melanoma and small cell lung cancer patients after vaccination with the anti-Id mAb BEC-2, although this occurred in a limited number of patients and was characterized as predominantly IgM [11,12].

The ability of 1E10 mAb vaccination to generate in humans Ab3 antibodies with the same antigenic specificity as P3 mAb (Ab1) [18,19] was demonstrated first in melanoma patients [20] and now has been confirmed in breast cancer patients, where serum antibodies reacting specifically against *N*-glycolyl-containing gangliosides were detected both by ELISA and HPTLC immunostaining. In addition, hyperimmune sera from breast cancer patients were able to specifically inhibit P3 mAb binding to NeuGc-GM_3, which confirmed that there were true Ab1′ antibodies in the patients. This antigen-specific response induced by 1E10 mAb was long lasting because high levels of anti-NeuGc-GM_3 antibodies were detected several months after patients finished the immune response induction phase of the treatment protocol. The application of a reimmunization schedule to some patients resulted in an increase in anti-NeuGc-GM_3 antibody titers. Although the maximum titer of the induction phase was not reached, repeated injections of 1E10 mAb vaccine every 3 months were safe and useful for maintaining high levels of Ag-specific antibody responses in patients.

Although NeuGc-containing gangliosides are widely expressed in most mammalian species, their presence in normal human tissues has not been reported so far. This difference could explain the ability of 1E10 mAb to induce antibody responses against the nominal Ag in immunized patients, in contrast to its incapacity to generate these kinds of antibodies in mice and monkeys [21].

An important finding of our study, previously observed in melanoma patients [20], was the detection of a relatively high level of binding of hyperimmune sera to NeuGc-GM_3 ganglioside, after the complete abrogation of the reactivity

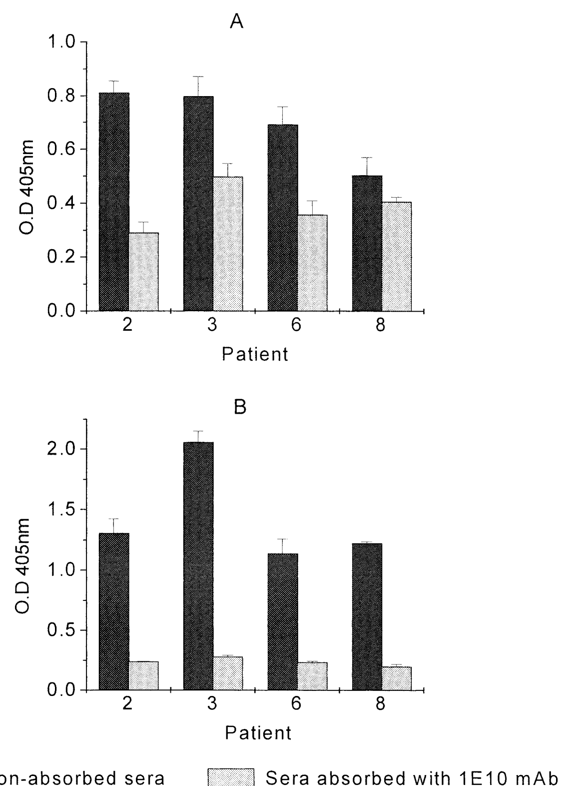

Fig. 9. Reactivity against NeuGc-GM_3 in breast cancer patients' sera absorbed with 1E10 mAb. Sera from patients were preincubated with 1E10 mAb and the remaining reactivity to NeuGc-GM_3 (A) and to 1E10 mAb (B) was evaluated by ELISA and compared to serum binding without preincubation with 1E10 mAb.

against 1E10 mAb by the absorption of patients' sera with this Ab2. This fact, together with the differences in the quality of the immunoglobulins generated against 1E10 mAb and NeuGc-GM_3, suggests the presence of antibodies characterized to be Id^-Ag^+. A similar finding was reported by Raychauduri et al. [36] when immunizing mice with an anti-Id mAb generated in the murine L1210 model; they detected the surprising presence of Id^-Ag^+ antibodies in the animals. In our case, these nonclassical antibodies could be produced by an unusual B cell population, different from the one producing the Id^+Ag^+ antibodies by classical antigen mimicry. The activation of this unusual B cells in breast cancer patients could be explained by an immunomodulatory effect exerted by 1E10 mAb, through idiotypic networks associated with the tumor. We suggest that a relaxation of natural tolerance to a tumor-associated ganglioside could be achieved by 1E10 mAb vaccine treatment. Although this hypothesis needs further demonstration, our group has accumulated experimental evidence that supports the idea of the involvement of P3 and 1E10 mAbs in B–T cell idiotypic networks [37]. The biological relevance of this nonclassical parallel set (Id^-Ag^+) remains to be elucidated.

In summary, 1E10 mAb vaccine has proved to be safe and highly immunogenic. A randomized phase II clinical trial is ongoing using the 1E10 vaccine to test whether the induction of a sustained immune response against *N*-glycolyl-containing gangliosides or glycoconjugates containing this type of sialic acid will improve the clinical outcome of breast cancer patients.

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