Characterization of the Antibody Response against NeuGcGM3 Ganglioside Elicited in Non-Small Cell Lung Cancer Patients Immunized with an Anti-Idiotype Antibody[1]

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1E10 mAb is an anti-Id murine mAb (Ab2 mAb) specific for an Ab1 mAb that reacts with NeuGc-containing gangliosides, sulfatides, and Ags expressed in some human tumors. In preclinical studies, this Ab2 Ab was able to mimic NeuGc-containing gangliosides only in animals lacking expression of these Ags in normal tissues. In this study, we report on the immune responses elicited in 20 non-small cell lung cancer patients treated with 1 mg of aluminum hydroxide-precipitated 1E10 mAb. In the hyperimmune sera from 16 of 20 patients, a strong specific Ab response of both IgM and IgG isotypes against NeuGcGM3 ganglioside was observed. Patient immune sera were able to induce complement-independent cell death of NeuGcGM3-expressing X63 murine myeloma target cells. Significant immunoreactivity to NeuGcGM3 was still detected after the complete abrogation of the reactivity against 1E10 mAb by the adsorption of patient sera with this Ab. We hypothesize that Id^-Ag^+ Abs could reflect the activation of an autologous idiotypic cascade into the patients. Both Id^+Ag^+ and Id^-Ag^+ fractions were separated by affinity chromatography and characterized. Although IgG isotype Abs were found in both fractions, IgM isotype Abs were found only in the Id^-Ag^+ fraction. Both Id^+Ag^+ and Id^-Ag^+ Abs were able to specifically recognize and induce cell death in NeuGcGM3-expressing X63 myeloma target cells. Patients that developed IgG and/or IgM Abs against NeuGcGM3 showed longer median survival times. *The Journal of Immunology,* **2008, 181: 6625–6634.**

Lung cancer is the leading cause of cancer-related mortality, with 1.2 million new cases worldwide diagnosed each year. The most frequent histological type is non-small cell lung cancer (NSCLC),[3] which constitutes ~80% of the total number of new cases (1, 2). Surgery is currently the only curative treatment for NSCLC, but because the majority of the patients are diagnosed with advanced disease this is seldom an effective course of action. The majority of NSCLC patients will require systemic chemotherapy, but unfortunately the median survival time even after the best available combination of systemic active drugs is limited to ~8–9 mo, and a 1-year survival rate is roughly 30–36% (3–7). These facts underscore the urgent need to develop new therapeutic approaches for NSCLC.

NeuGc-containing gangliosides are attractive targets for cancer immunotherapy as these glycolipids are not normally expressed in humans and are therefore foreign Ags, but they have been detected in a range of human tumors by Abs and chemical analysis (8–12). Additionally, recent experimental data suggest that NeuGcGM3 is relevant for tumor progression (13). One strategy to generate immune responses to these glycolipids is the use of anti-Id Abs (Ab2). This approach arose from Jerne's idiotypic network theory (14), which postulates that, due to the large potential diversity of Ig variable regions, the Id repertoire can mimic the universe of self and foreign epitopes. Thus, properly selected anti-idiotypic Abs could act as tumor-associated ganglioside surrogates. In addition to our own experience, two other anti-idiotypic mAbs mimicking gangliosides have been used in clinical trials with cancer patients, but with limited results (15, 16).

We previously reported a vaccine preparation featuring a murine anti-Id mAb related to the NeuGc-containing ganglioside Ag model. This Ab2, named 1E10 (17), was generated from the immunization of BALB/c mice with P3, an idiotypic Ab (Ab1) that recognizes NeuGc-containing gangliosides, sulfated glycolipids, and Ags present in different human tumors including those from the lung, and which contains a regulatory Id according to Bona's concept (18–24). Preparations containing 1E10 mAb were able to induce antitumor effects against lung metastases in murine models, and phase I clinical trials have proven the safety and immunogenicity of 1E10 Id vaccination in melanoma and breast cancer patients (20, 25, 26). From these latter studies, high titer Ab responses to NeuGc-containing gangliosides were measured in the sera of cancer patients. A fraction of non-suppressible anti-NeuGc-containing ganglioside Abs was demonstrated through adsorption of these sera with 1E10 mAb, suggesting that 1E10 Id vaccination might enhance antitumor natural immune response (20, 26). Furthermore, NeuGcGM3-specific IFN-γ-secreting cells were measured by ELISPOT from PBMC of 1E10-vaccinated breast cancer patients (27).

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Received for publication January 8, 2008. Accepted for publication August 20, 2008.

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[1] This work was supported by Recom-Bio S.L. and by the Cuban Government.

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[3] Abbreviations used in this paper: NSCLC, non-small cell lung cancer; Ab2, anti-Id Ab; Ab1, idiotypic Ab; HPTLC, high performance TLC; PI, propidium iodide; CI, confidence interval.

Table I. *Number of doses received, maximal titer, and isotype analyses against 1E10 mAb and NeuGc-GM3, and survival times in vaccinated NSCLC patients*

Patients	No. of Doses	Title vs 1E10 mAb		Title vs NeuGcGM3		Survival (mo)
		IgM	IgG	IgM	IgG	
01	5		1/25600	1/6400	1/1600	6.5
02	19		1/6400	1/1600	1/800	17.3
03	18		1/12800	1/3200	1/800	17.3
04	20		1/25600	1/3200	1/800	17.3
05	7					4.97
06	12		1/3200		1/800	13.33
07	20		1/25600	1/25600	1/1600	17.07
08	9		1/12800	1/12800	1/12800	9.93
09	11		1/12800	1/1600	1/800	9.3
10	12		1/12800	1/3200	1/1600	11.73
11	11					6.1
12	20		1/6400	1/3200	1/1600	17.07
13	11		1/3200			9.67
14	18		1/25600	1/6400	1/3200	16.13
15	19		1/6400	1/1600	1/800	15.2
16	9		1/6400	1/1600	1/3200	5.97
17	9		1/12800	1/3200	1/6400	8.87
18	8		1/1600			6.6
19	19		1/3200	1/400	1/3200	15.4
20	7		1/6400	1/400		5.97

More recently we performed a clinical study in NSCLC patients that showed encouraging clinical benefits (28). We now report the immune response elicited in 20 advanced NSCLC patients treated with 1E10 mAb, and demonstrate the induction of Abs that are Id^+Ag^+, but also Id^-Ag^+. Both Ab fractions recognized and induced the death of myeloma cells expressing NeuGcGM3 by a complement-independent mechanism. Those patients who developed IgG and/or IgM Abs against NeuGcGM3 showed a longer survival time.

We hypothesize 1E10 Id vaccination could induce an idiotypic cascade, which would amplify the Ag-specific immune response to a tumor-associated neo-self ganglioside Ag. This therapeutic concept goes well beyond the classical concept of Ag mimicry.

Materials and Methods

Gangliosides and cells

Gangliosides NeuAcGM3 and NeuGcGM3 purified from dog and horse erythrocytes, respectively, as described earlier (29), were provided by Dr. L. E. Fernández (Vaccine Department, Center of Molecular Immunology, Havana, Cuba). P3-X63-Ag8.653 (X63) murine myeloma cell line (ATCC NCRL 1580), expressing NeuGcGM3 in their membrane (30), and H82 human lung carcinoma cell line (ATCC HTB-175), negative for the expression of the ganglioside, were grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS (HyClone), 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and maintained at 37°C with 5% CO2.

Anti-Id Ab (1E10)

Ab2 1E10 mAb (IgG1, κ) was generated by immunizing BALB/c mice with P3 mAb (IgM, κ; Refs. 17, 18). 1E10 mAb was purified from ascites, and the aluminum hydroxide-precipitated mAb vaccine was produced in accordance with the Good Manufacturing Practice guidelines and certified by the Quality Control Department of the Center of Molecular Immunology, as previously reported (20).

Patients

Twenty patients with histo- or cytological confirmed advanced NSCLC were eligible for enrollment in a compassionate use study, after providing written, informed consent. All the patients have previously received the oncospecific treatment established in the Oncological Therapeutic Standards, according to their stage at the moment of the diagnosis by the National Comprehensive Cancer Network guidelines (version 2.0, 2006). First line chemotherapy, consisting mainly on cisplatin/vinblastin, had to be completed 4 wk before the patients entered the study. Other eligibility criteria included World Health Organization performance status $\leq$2, age $\geq$18 years, normal hematopoietic, hepatic, and renal functions, and life expectancy higher than 3 mo. The most important exclusion criteria included the presence of brain metastases, pregnancy or lactation, serious chronic diseases, and active infections. The study was approved by the Institutional Review Board of the hospital where the study was developed.

Treatment schedule

Patients were injected intradermally with 15 doses of 1 mg of aluminum hydroxide-precipitated 1E10 mAb, as base treatment. The first five doses were administered every 14 days, and the remaining 10 doses were administered every 28 days. After 15 doses, reinmunizations were administered at 28-day intervals, if the patients maintained a favorable clinical status. Serum was obtained before and during treatment. Patients who received one or more vaccine doses were evaluable for toxicity and clinical results, and those who received at least four doses of

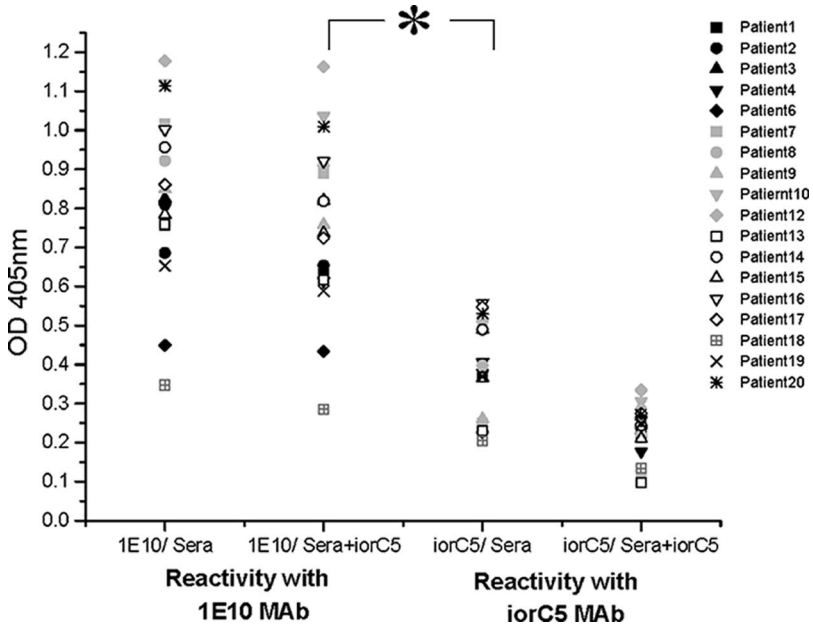

FIGURE 1. Immunodominance of 1E10 mAb Id in the Ab response induced in vaccinated NSCLC patients. Hyperimmune sera from patients immunized with aluminum hydroxide-precipitated 1E10 mAb were preincubated with the isotype-matched ior C5 mAb, and later the reactivity against 1E10 and ior C5 mAbs was assessed by ELISA. *, $p < 0.01$, Mann-Whitney U test, one-tailed.

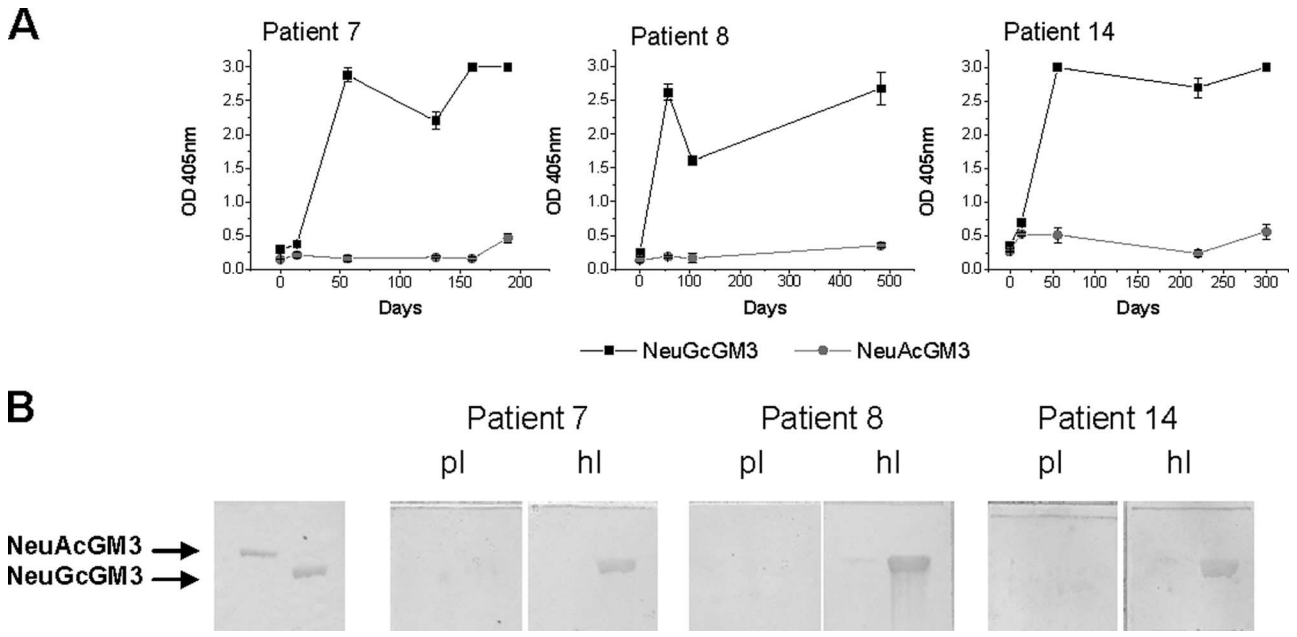

FIGURE 2. Kinetics and specificity of Ab3 response against NeuGcGM3 in the sera of NSCLC. *A*, Sera from vaccinated patients diluted 1/400 were bound to microtiter plates coated with NeuGc and NeuAcGM3 (200 ng/well), and the reaction was developed with biotinylated goat anti-human IgG $^+$ IgM, followed by the addition of alkaline phosphatase-streptavidin complex. *B*, Gangliosides (1 μg) were chromatographed with chloroform:methanol:0.2% $CaCl_2$ in 2.5 M NH3 (5:4:1; v/v/v) and visualized with orcinol. For the immunostaining, the plates were incubated with the patients preimmune (pI) and hyperimmune (hI) sera, diluted 1/200, and the reaction was developed with alkaline phosphatase conjugated goat anti-human IgG $^+$ IgM.

aluminum hydroxide-precipitated 1E10 mAb were considered immunologically evaluable.

Measurement of Ab response

To measure Ab3 reactivity against 1E10 mAb and purified gangliosides in sera from NSCLC patients, solid-phase ELISAs were performed as previously described (20). The highest serum dilution giving OD values $\geq$0.25 and being at least three times the value corresponding to the preimmune serum at the same dilution was considered as titer. Assays were performed in triplicate for each sample and the coefficient of variation was $<$15%. The OD of the blanks was $<$0.1. The presence of Abs specific to gangliosides was detected by immunostaining on high performance TLC (HPTLC) plates, and by ELISA, as previously described (20).

Ab-binding inhibition assays

To define the extent of the Id-specific response against 1E10 mAb, patients' hyperimmune sera were incubated overnight at 4°C with isotype-matched irrelevant mAb ior C5, specific for a glycoprotein expressed on human colorectal cells (31) at a final concentration of 0.5 μg/ml, to adsorb the human Abs against the isotypic determinants of 1E10 mAb. Then, samples were added onto 1E10 mAb-coated plates and remnant reactivity was assessed by the ELISA procedures previously described (20). Ior C5 mAb-coated plates were used to measure the isotypic response in non-adsorbed sera and as controls of the absorption efficiency.

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 at a final concentration of 0.5 μg/ml, and added onto plates coated with NeuGcGM3. Serum reactivity was assessed by the ELISA procedures previously described (20). ELISA plates coated with 1E10 mAb were used as a control of the adsorption efficiency.

Isolation of Id positive and negative Ab fractions

Hyperimmune sera from NSCLC patients and their preimmune sera, used as negative control, were diluted 1/2 with PBS to a final volume of 200 μl and incubated with 100 μl of Sepharose 4B-coupled 1E10 mAb matrix, overnight, at 4°C with shaking. The supernatant was recovered and Abs bound to the column were eluted with 200 μl of glycine-HCl (pH 2.8) and neutralized with 2 M Tris. Reactivity of the eluted and unbound fractions against 1E10 mAb and gangliosides was tested by ELISA as previously described (20).

Flow cytometry

Patient serum samples or Ab fractions were incubated with 5×10^5 X63 cells for 30 min on ice. After washing, the cells were incubated with FITC-conjugated goat anti-human immunoglobulins (Jackson ImmunoResearch Laboratories) for 30 min on ice. H82 lung carcinoma cells were used as negative control. To determine whether the reactivity of the isolated Id^- and Id^+ Abs against the cells was not due to the presence of remnant Abs reacting with 1E10 mAb, both fractions were preincubated with 1E10 mAb at a final concentration of 0.5 μg/ml overnight at 4°C. Percentage of positive stained cells were determined in a FACScan instrument (BD Biosciences). The WinMDI 2.8 444 program was used to analyze a total of 10^4 cells acquired on every FACS assay.

Induction of cell death

Patients' sera or Ab-purified fractions were incubated with 2×10^5 X63 cells in 100 μl of RPMI 1640 culture medium supplemented with 1% FCS in 5% CO_2 atmosphere at 37°C for 4 h. The cell death induction was detected by the addition of propidium iodide (PI; Sigma-Aldrich) at a final concentration of 10 μg/ml and analyzed by flow cytometry. Similar experiments were performed with patients' samples previously heated 30 min at 56°C for complement inactivation.

Statistical analysis

The mean value and the SD of the values obtained in the triplicates of each sample were calculated. Each experiment was repeated at least twice. The mean values and the SDs were plotted using the Microcal Origin program. Mann-Whitney *U* test was used as a non-parametric test for pair-wise comparisons. Survival times were estimated using the Kaplan-Meier method via the SPSS Program, version 10, and the differences in the median survival times between responders and non-responders were compared using the log-rank test.

Results

1E10 mAb induced a specific Ab response against its Id (Id^+) in NSCLC patients

NSCLC patients were considered as immunologically evaluable when they had received at least five doses of the aluminum hydroxide-precipitated 1E10 mAb. Ab responses induced by immunization with 1E10 mAb were tested in sera obtained from patients before and during the treatment. Of the 20 patients, 18

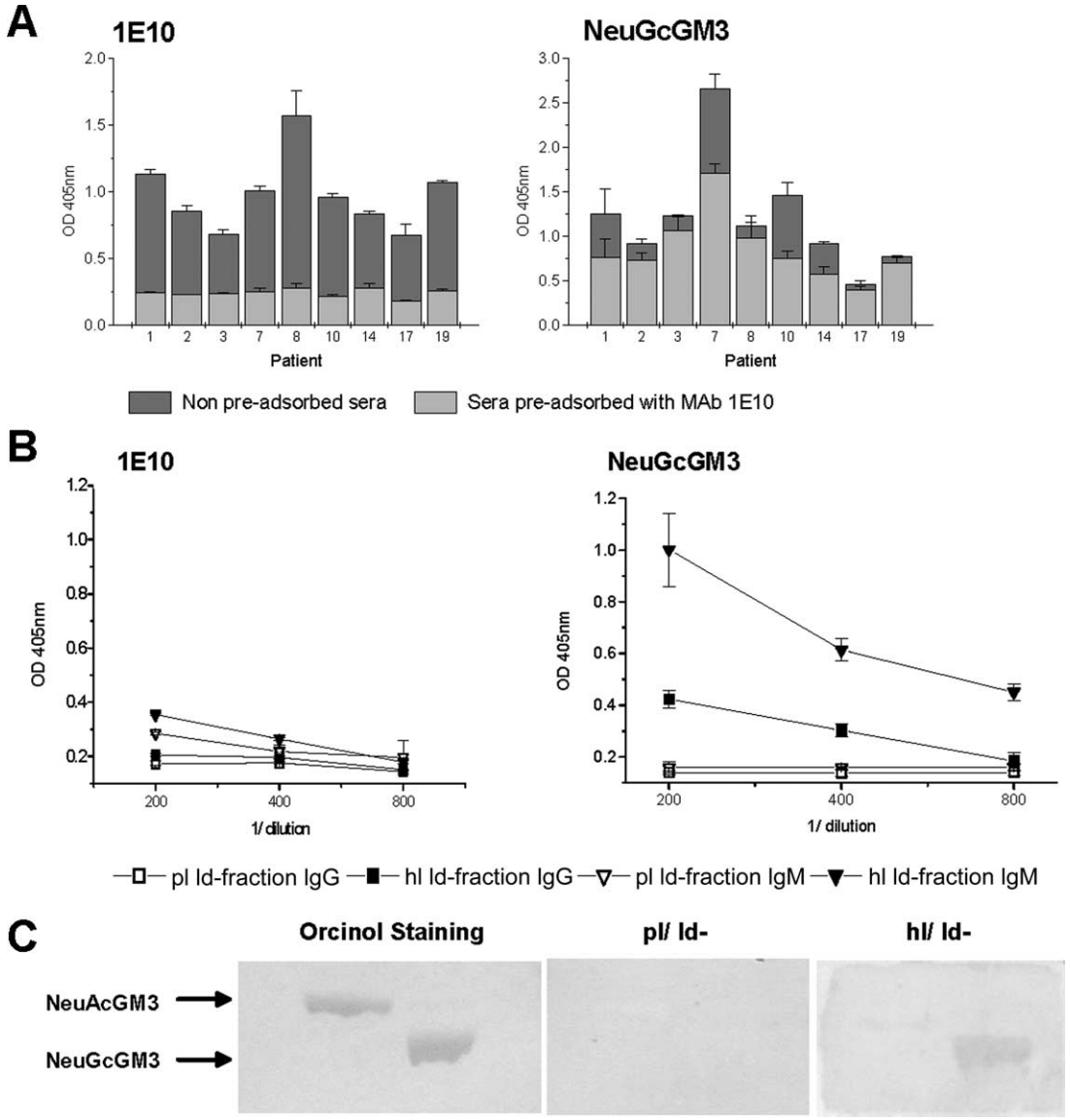

FIGURE 3. Reactivity against NeuGcGM3 in patient's sera absorbed with 1E10 mAb. *A*, Sera from NSCLC patients were preincubated with 1E10 mAb and the remnant reactivity to 1E10 mAb and NeuGcGM$_3$ was assessed by ELISA. *B*, Id$^-$ Ab fraction isolated from patient 7 was incubated with 1E10 mAb or NeuGcGM3 on ELISA plates. *C*, Gangliosides (1 μg) were chromatographed with chloroform:methanol:0.2% $CaCl_2$ in 2.5 M NH3 (5:4:1; v/v/v) and visualized with orcinol. For the immunostaining, the plates were incubated with the Id$^-$ Ab fractions isolated from the preimmune (pI) and hyperimmune (hI) sera of patient 7, diluted 1/200, and the reaction was developed with alkaline phosphatase-conjugated goat anti-human IgG $^+$ IgM.

developed Abs against 1E10 mAb. This Ab response was of the IgG isotype with titers ranging from 1/3,200 to 1/25,600 (Table I); no IgM Abs were detected at the lowest serum dilution tested (1/100).

To confirm that a specific response against 1E10 mAb Id was generated by the immunization, patients' hyperimmune sera were preabsorbed with the isotype-matched control mAb, and the remaining reactivity against 1E10 mAb was measured by ELISA. As shown in Fig. 1, strong reactivity against 1E10 mAb was detected in patient sera after preabsorption with the control mAb. The level of Ab response against 1E10 mAb Id was higher than the reactivity of the non-preadsorbed sera against the isotype in all patients ($p < 0.05$, Mann-Whitney *U* test, one tail).

1E10 mAb immunization induced a specific Ab response against NeuGcGM3 in the patients (Ag$^+$)

Pre- and postimmunization sera samples were tested by ELISA for the recognition of NeuGcGM3 and NeuAcGM3 to determine whether the treatment of NSCLC patients with 1E10 mAb induced Abs with the same specificity of P3 mAb (Ab1). Sixteen patients developed Abs of IgM and/or IgG isotype that specifically bound to NeuGcGM3. No reactivity to NeuAcGM3was detected (Fig. 2*A*). Titers of up to 1/25,600 and 1/12,800 of IgM and IgG responses were obtained, respectively (Table I). Analysis of the isotype of the anti-NeuGcGM3 response indicated that 14 patients generated both IgG and IgM Abs, one patient only showed IgG, and in another patient only IgM Abs specific to NeuGcGM3 were detected. The Ab response against NeuGcGM3 ganglioside was increased with the course of vaccination, reaching a peak after patients received the fourth or fifth doses of the anti-idiotypic mAb. The specificity of the anti-ganglioside Ab response was confirmed by HPTLC immunostaining where an evident specific binding of hyperimmune patients' sera with NeuGcGM3 was observed. No reaction was detected when preimmune patients' sera were tested (Fig. 2*B*).

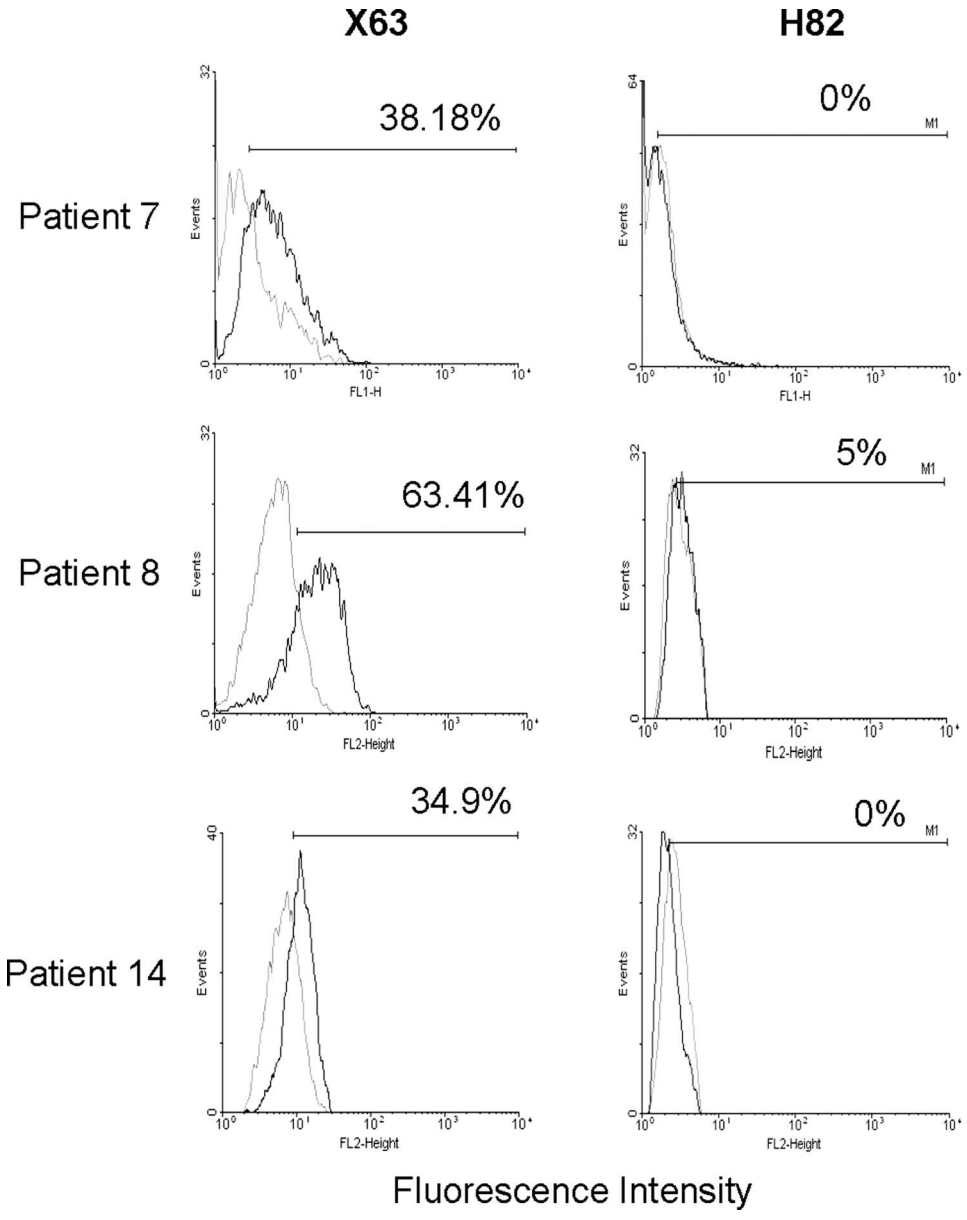

FIGURE 4. Binding of preimmune and hyperimmune sera of 1E10 mAb-treated NSCLC patients to myeloma cell line P3-X63-Ag8.653. Patients preimmune (light gray) and hyperimmune (black) sera, diluted 1/10, were incubated with the NeuGcGM3 expressing myeloma cell line P3-X63-Ag8.653 or the control human cell line H82. The reaction was developed with FITC-conjugated anti-human IgG^+IgM. The numbers represent the percentage of hyperimmune sera-reacting cells after the subtraction of the value of preimmune sera-reacting cells.

1E10 mAb immunization generated Ag^+Id^+ and Ag^+Id^- Abs in the immunized patients

As 1E10 mAb immunization generated different isotype pattern Ab responses against the mAb molecule and the ganglioside, we studied whether 1E10 mAb induced the activation of NeuGcGM3-related idiotypic networks through the detection of Abs characterized to bind to NeuGcGM3 and not to 1E10 mAb (Ag^+Id^-) in patients' sera. To accomplish this, hyperimmune patient sera were preincubated with saturating amounts of 1E10 mAb, and the remaining reactivity against NeuGcGM3 was measured by ELISA. Plates coated with 1E10 mAb were used as controls of the adsorption efficiency, showing that there was no binding with 1E10 mAb by the preadsorbed sera (Id^-). Significant binding activity to NeuGcGM3 was still detected in preabsorbed sera of all the patients studied, suggesting the presence of a fraction of Abs characterized to be Ag^+Id^- (Fig. 3*A*). To further characterize these Abs, the Id^- fraction was separated from the Id^+ fraction by incubating hyperimmune patient sera with a Sepharose 4B-coupled 1E10 mAb matrix. The bound (Id^+) and the unbound (Id^-) Ab fractions were then recovered, and their reactivity against 1E10 mAb and NeuGcGM3 was confirmed by ELISA using fractions isolated from preimmune sera as negative controls. As is shown in Fig. 3*B*, no binding with the 1E10 mAb could be detected in the Id^- fraction. However, this fraction contained not only IgM, but also IgG Abs against NeuGcGM3. The specificity of these Abs was further corroborated by HPTLC immunostaining, where Id^- Abs reacted with NeuGcGM3 but not with the *N*-acetylated variant of the ganglioside chromatographed on the TLC plate (Fig. 3*C*).

We next evaluated the capacity of patient sera to recognize naturally expressed NeuGcGM3 on tumor cells. To do this, preimmune and hyperimmune sera of the patients were incubated with the myeloma cell line X63, which expresses high levels of NeuGcGM3 (75% of all glycolipids expressed at the cell membranes; Ref. 30), and the binding was measured by flow cytometry. Hyperimmune sera from 10 of the 12 patients tested showed significant binding to X63 myeloma cells compared with their preimmune ones. In contrast, both pre- and hyperimmune sera from patients showed very low reactivity to the NeuGcGM3 negative H82 cells. Fig. 4 shows the results

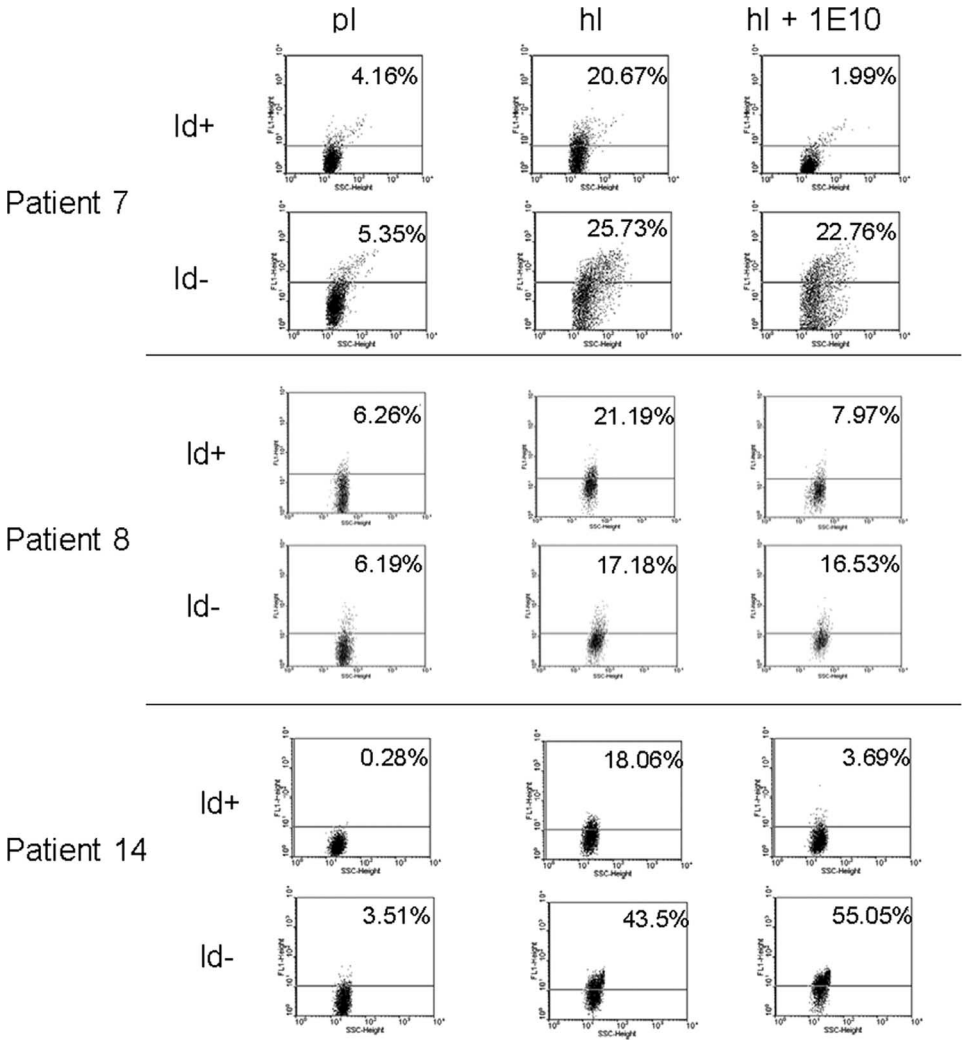

FIGURE 5. Binding of Id^+ and Id^- Abs to the myeloma cell line P3-X63-Ag8.653. Id^- and Id^+ Abs, diluted 1/10, were preincubated or not with saturating amounts of 1E10 mAb, and the binding with the myeloma cell line P3-X63-Ag8.653 was detected by flow cytometry with FITC-conjugated anti-human IgG^+IgM. Numbers represent the percentage of labeled cells.

obtained with the sera from three representative patients. To determine which fractions (Id^+ or Id^-) were responsible for this recognition, the binding of each to the X63 myeloma cells was studied. As shown in Fig. 5, both Ab fractions obtained from hyperimmune patients' sera bound to the myeloma cells, and a very low percentage of the cells was recognized by the Ab fractions isolated from preimmune sera. The reactivity of the Id^- Ab fractions obtained from hyperimmune sera was not due to the presence of remnant Abs reacting with 1E10 mAb at the dilution tested, because preincubation of these fractions with the anti-Id mAb did not inhibit their binding. In contrast, the adsorption of the Id^+ Ab fractions with 1E10 mAb abrogates their recognition of the myeloma cells (Fig. 5). These results together indicate that 1E10 mAb treatment induced in the patients the generation of Abs specific to NeuGcGM3 due to their recognition of 1E10 Id, and also of Abs specific to the nominal Ag, but not to the anti-Id mAb.

1E10 mAb generated Abs capable of inducing cell death

With the objective to study whether the Abs developed in the immunized patients were able not only to recognize, but also to kill X63 myeloma cells, patients' sera were incubated for 4 h at 37°C with the cells, and the effect on their viability was studied by flow cytometry using the PI exclusion assay. In 9 of 15 patients studied, an increase in PI incorporation in the cells incubated with hyperimmune patient sera was observed over that caused by preimmune sera (Fig. 6). The results shown in Fig. 6 suggest that this cell death was induced by a mechanism independent of complement cascade activation, because it was not inactivated by heating the sera (30 min at 56°C) before use in the assay. We next studied whether both Id^- and Id^+ Ab fractions also possess this cytotoxic capacity by incubating them with the myeloma cells, as previously described. As is shown in Fig. 7, both Id^- and Id^+ Abs were able to induce the death of the cells. These results suggest that 1E10 mAb treatment induced in the patients the generation of Abs with the potential to recognize and kill tumor cells expressing NeuGcGM3.

1E10 mAb-immunized patients who developed anti-NeuGcGM3 Abs had longer survival times

Patients enrolled in the study were evaluated for safety. The overall toxicity of 1E10 anti-Id vaccine was classified as grade 1 and 2, according to the National Cancer Institute Common Toxicity Criteria (version 3.0). The most common side effects were local reaction in the injection site with erythema and induration, occasionally associated with mild pain that disappeared in a few days (24–72 h). Some patients had fever, pruritus, arthralgias, and mild headache. All the symptoms were independent of the number of doses administered, and they lasted between 1 and 3 days and disappeared spontaneously. No other severe or unexpected adverse events were observed, and neither biochemical nor hematological abnormalities were reported.

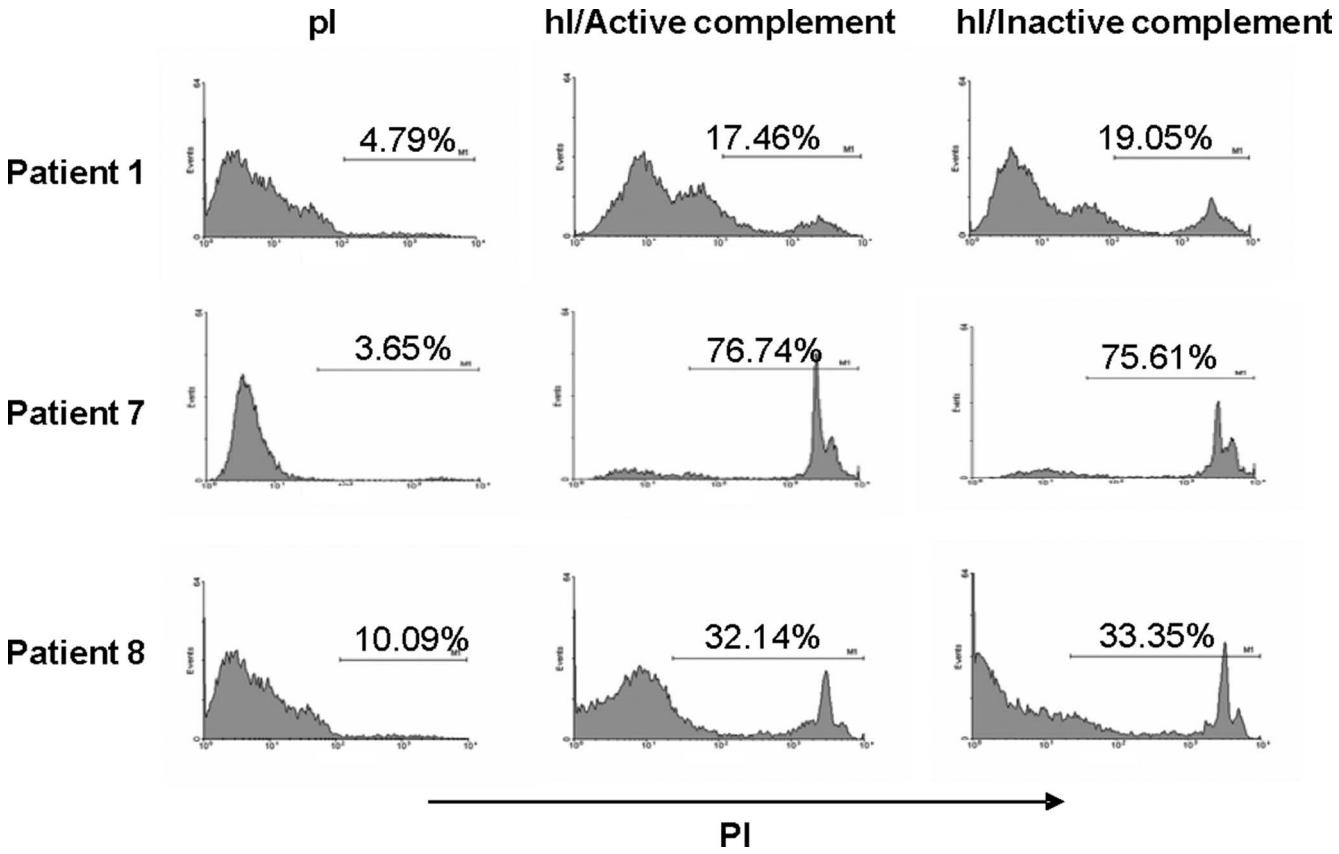

FIGURE 6. NSCLC vaccinated patient's sera induced cell death to the myeloma cell line X63 by a mechanism independent of complement cascade. X63 cells were incubated for 4 h at 37°C with patients 1, 7, and 8 preimmune (pI) and hyperimmune (hI) sera, diluted 1/10, without any treatment (Active complement) or previously inactivating the complement cascade by heating (Inactive complement). The percentage of cell death was determined by the PI incorporation assay.

Table I shows the individual survival of the NSCLC patients that participated in this study. The overall median time survival of the patients treated with 1E10 mAb was 10.6 mo (95% confidence interval (CI), 3.0–17.3 mo). There were statistically significant differences between the median survival time of the patients that developed IgM and/or IgG Abs against NeuGcGM3 (median survival time 14.26 mo; 99% CI, 5.97–17.3 mo) and the median survival time of the patients that did not develop Abs against the ganglioside (median survival time 6.35 mo; 95% CI, 4.97–9.67 mo; $p < 0.01$, log-rank; Fig. 8).

Discussion

In this study we characterize the immune response against NeuGcGM3 ganglioside induced in NSCLC patients due to the immunization with the anti-Id 1E10 mAb. A predominant IgG Ab response against 1E10 mAb Id was developed in most of the immunized patients. The Id immunodominance was operationally defined by incubating patient sera with an irrelevant mAb to adsorb the reactivity against the murine isotype and then measuring the remaining immunoreactivity against 1E10 mAb and comparing it to the anti-isotype response. The magnitude of the remaining anti-Id response was significantly higher than the response detected in non-adsorbed sera against the control Ab (anti-isotype response; Fig. 1). This Id immunodominance was observed not only in this study, but also in two previous phase I clinical trials conducted in advanced melanoma and breast cancer patients (20, 26). Moreover, similar results were obtained when monkeys and chickens were immunized with 1E10 mAb (32), suggesting that 1E10 mAb Id immunodominance is not a species-depending property. The 1E10 mAb Id immunodominance is a characteristic specially important for a murine Ab that is devoted to use for cancer patient treatment, because it could avoid the induction of a high immune response against the murine isotype, which in some cases can generate a diminishment of the treatment efficacy and adverse reactions that usually worsen with the increase of the dose number (33, 34).

A low frequency of side effects was observed in our study. Thirteen patients received more than 10 doses of the anti-Id mAb, and confirmation of the safety of the treatment with this anti-Id vaccine preparation was reported in the previous clinical trials (21, 26, 27). The treatment of NSCLC patients with 1E10 mAb elicited Abs that shared the capacity of P3 mAb to recognize NeuGcGM3. The presence of these Ag^{+}-specific Abs was demonstrated by direct binding to the purified ganglioside assessed by ELISA and TLC immunostaining, and by their recognition of the NeuGcGM3-positive myeloma cell line X63 by flow cytometry. In most of the patients we detected a relatively high titer of anti-NeuGcGM3 Abs of both IgM and IgG isotypes. This is a relevant result taking into account that is difficult to obtain an IgG Ab response against these Ags (35). Even the use of anti-Id Abs as protein mimicries of gangliosides does not guarantee the induction of this kind of response. Previously, it was reported that most of the melanoma patients immunized with 1A7 mAb able to mimic GD2 ganglioside developed specific IgG Abs against this ganglioside (15). In contrast, when melanoma and small cell lung cancer patients were treated with the anti-Id BEC-2 mAb, the percentage of patients that developed anti-GD3-specific Ab response was low, mainly of IgM isotype. The presence of these Abs was detected by ELISA, but could not be confirmed by TLC immunostaining or flow cytometry

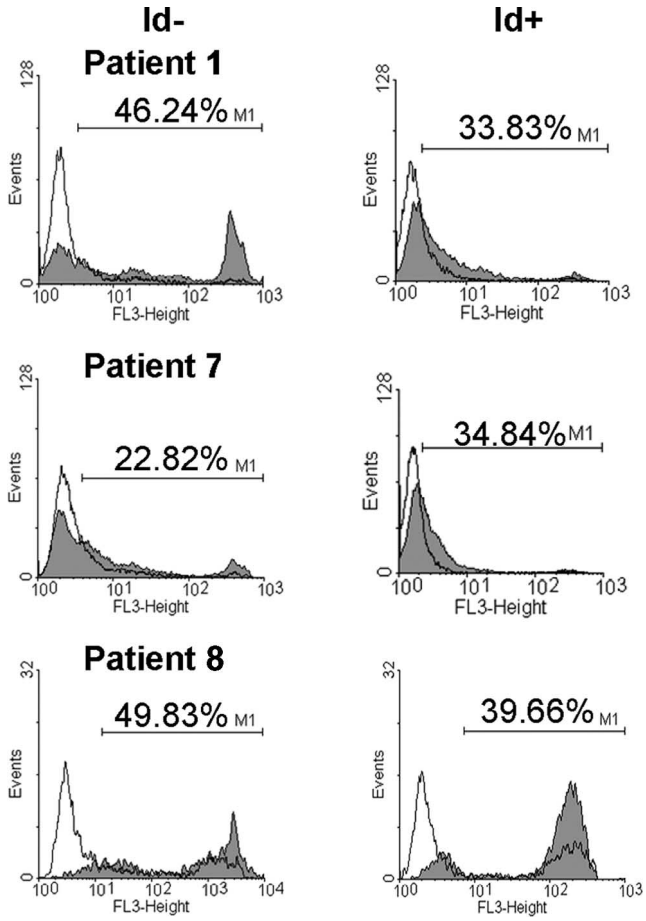

FIGURE 7. The Id^+Ag^+ and Id^-Ag^+ fractions isolated from NSCLC-vaccinated patient's sera induced cell death to the myeloma cell line X63. Cells were incubated for 4 h at 37°C with the Id^- and Id^+ Ab fractions isolated from patient 1, 7, and 8 preimmune (bold line) and hyperimmune (shaded area) sera, diluted 1/10. The percentage of cell death was determined by the PI incorporation assay.

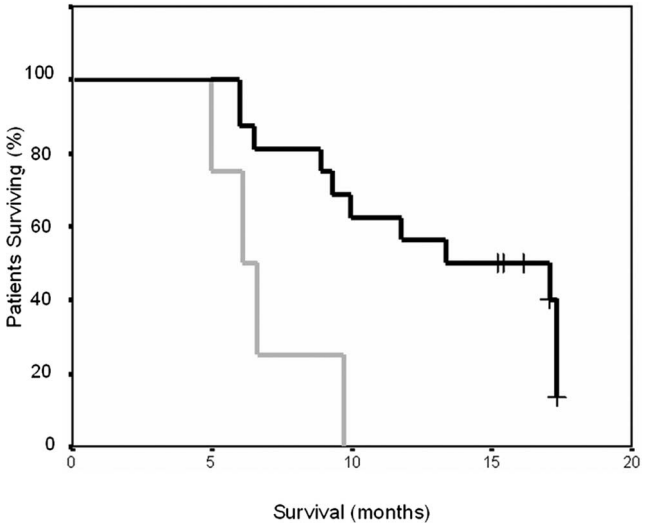

FIGURE 8. Kaplan-Meier survival curves of patients that developed IgM and/or IgG Abs against NeuGcGM3 (black) and of the patients that did not develop Abs against the ganglioside (gray; $p < 0.01$, log-rank). On the *y*-axis, the percentage of surviving is reported; on the *x*-axis, the time from entrance the study (months) is reported.

(36, 37). The differential induction of Ab responses against gangliosides could be dependent on their different expression in normal tissues. In fact, studies previously reported showed the relation between the level of ganglioside expression in human and murine normal tissues and their immunogenicity (38–40).

The precise cellular and molecular mechanisms that mediate tolerance against gangliosides have not yet been elucidated. Indirect evidence suggests that B2 cell repertoire is regulated, avoiding the generation of a mature Ab response against these Ags (38).

The strong Ab response against NeuGcGM3 induced in patients by the 1E10 mAb can be explained because NeuGc-containing gangliosides are not self-Ags in humans, as the gene for the enzyme responsible for NeuGc biosynthesis is inactivated (41–43). The very small amounts of NeuGc conjugates detected in some human normal tissues appear to originate from exogenous sources (44, 45). One explanation for the higher expression of NeuGc-containing gangliosides in some tumors is the recent demonstration that tumor hypoxia induces the transcription in the cells of a sialic acid transporter that facilitates the incorporation of NeuGc molecule into tumor gangliosides, as NeuGcGM2 (46). It is noteworthy that our previous preclinical studies proved that 1E10 mAb can behave as an immunogenic mimic, depending on the presence or absence of NeuGcGM3 ganglioside in the normal tissues of the immunized species (32).

The different isotype pattern in the response against 1E10 mAb molecule, where only IgG Abs were detected, in comparison with the IgG and IgM Ab response against NeuGcGM3, suggested that different B cell populations could be activated in the patients to produce Abs against this ganglioside. An important finding of this study was the detection of a high level of non-suppressible immune reactivity to NeuGcGM3 ganglioside by the adsorption of patient sera with 1E10 mAb. A similar finding in preclinical studies was reported by Lange and Lemke in mice immunized with an Ab against phenyl-oxazolone (47), and by ourselves in chickens immunized with 1E10 mAb (32). The presence of these Id^-Ag^+ Abs due to the immunization of NSCLC patients with 1E10 mAb also confirm the results obtained in advanced melanoma and breast cancer patients treated with this anti-Id mAb (20, 26).

Our studies included a fractionation of the patient serum samples to further characterize the Id^-Ag^+ Abs. We then isolated an Id^-Ag^+ Ab-enriched fraction from patient immune sera by removing the Id^+ Ab fractions with 1E10 mAb-coupled to Sepharose 4B matrix. Their reactivity against NeuGcGM3 and not against 1E10 mAb was demonstrated by ELISA (Fig. 3*B*). To our knowledge, there is no other evidence to date supporting the induction of Id^+Ag^+ and Id^-Ag^+ Abs related to a ganglioside-specific immune response in cancer patients.

The formation of immune complexes between tumor ganglioside Ags and the induced Ab3 (Id^+Ag^+) could eventually amplify the specific anti-ganglioside Ab response, either by improving Ag presentation somehow or by modifying the antigenicity of such tumor gangliosides in the context of the immune complexes, providing an alternative explanation to the induction of the Id^-Ag^+ Abs. However, this explanation is not likely true for the Id^-Ag^+ Abs detected in our study, because they, like Ab3 Abs, were highly specific for NeuGcGM3, being able to discriminate between this ganglioside and the acetylated version (NeuAcGM3). The only difference between these two gangliosides is a single oxygen atom at the *N*-acetyl moiety of the sialic acid, which is involved in the interaction between NeuGcGM3 and Ab3 Abs. Then we should expect a different epitope recognition pattern for Abs induced by the putative immune complexes.

Preclinical data already published by our group suggest that P3 and 1E10 mAb could be able to activate idiotypic networks, involving both B and T cells. Pérez et al. (23) showed that lymph node cells from BALB/c mice immunized with P3 mAb proliferated in vitro, in a dose-dependent manner, not only in response to P3 mAb but also to 1E10 mAb, suggesting the existence of a naturally occurring B-T cell idotypic network. Rodríguez et al. (48) showed that chickens immunized with P3 mAb (Ab1) or 14F7 mAb (Ab1), another Ab specific for NeuGcGM3, developed an anti-idiotypic response against both immunizing Abs. Only those chickens, however, immunized with P3 mAb were able to develop a strong and specific Ab response against *N*-glycosylated gangliosides. The detection of Abs with this specificity in animals immunized with an Ab1 suggested that the elicited Ab2 Abs behaved in vivo as a ganglioside surrogate inducing a specific Ab3 response against these Ags.

This previous evidence and the immunochemical results presented in this study suggest that the vaccination of cancer patients with 1E10 mAb could induce the activation of an idiotypic cascade. Although any idiotope could be able to mimic any Ag, only those related to Ags that have been fixed by evolution, due to their relevance for organism homeostasis, will be immunodominant and capable of inducing natural idiotypic cascades (49). Among the available Ab3 idiotopes induced by 1E10 mAb immunization in our model, those related to the NeuGcGM3 binding site could be recognized by Ab4 natural Abs in a different way than by 1E10 mAb and may possess a different type of mimicry. These kind of Ab4 Abs might then induce NeuGcGM3-specific Ab5 Abs that do not recognize 1E10 mAb Id (Id^-Ag^+). The involvement of T cells in the activation of idiotypic Ab cascades has been reported previously (23, 50), and the existence of these anti-idiotypic T cells in our case could explain the induction of specific IgG Abs against NeuGcGM3.

At present, most of the Id vaccine approaches are based exclusively in the mimetic capacity of the anti-Id Abs, without searching for their immunoregulatory potential. The use of anti-Id Abs as immunogens could offer the possibility not only of generating Ab3 Abs against their own idiotopes, but also inducing a cascade of Id-anti-Id interactions leading to an amplified and long lasting immune response against the nominal Ag. The expansion of natural Ab repertoire by Id vaccination could even participate in the lysis of tumor cells. In fact, recent reports showed that natural IgM Abs that detect specific structures on aberrant cells can remove these cells by inducing apoptotic stress (51–53).

One question we wanted to address was whether the generation of NeuGcGM3-specific Abs in patients could have some biological impact in tumor cells expressing this ganglioside. Our results from the flow cytometry studies showed that most of the patients' sera tested bound to NeuGcGM3-positive myeloma cells and were killed by a complement-independent mechanism. Furthermore, we demonstrate that the Id^+ and the Id^- fractions isolated from patients' hyperimmune sera are capable of inducing this cytotoxic effect. We do not know yet which cytotoxic mechanism is activated by these Abs. One possibility could be that they mediate mechanisms of programmed cell death, because it has been reported for other Abs specific to non-protein Ags, like gangliosides (54–57).

Several clinical trials using anti-Id Abs in cancer patients have referred a correlation between a better clinical response and the induction of Abs against the nominal Ag (15, 58–60). Thus, we evaluated whether the Ab response developed in NSCLC patients due to 1E10 mAb immunization had clinical relevance. The results of the analysis showed that there was a relation between longer survival times and the induction in the patients of anti-NeuGcGM3 Abs. A randomized, double blind phase II clinical trial is ongoing to evaluate the clinical effect of 1E10 mAb vaccine in NSCLC and to define the value of the Abs induced by the anti-Id treatment as real predictors of clinical outcome.

Acknowledgments

We thank Dr. Stephen P. Schoenberger for critically reviewing the manuscript. We also thank Dr. L. E. Fernández for generously providing gangliosides.

Disclosures

The authors have no financial conflict of interest.

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