Immunotherapy of Advanced Breast Cancer With a Heterophilic Ganglioside (NeuGcGM3) Cancer Vaccine

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Purpose: A heterophilic ganglioside cancer vaccine was developed by combining NeuGcGM3 with the outer membrane protein complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP). A phase I clinical trial was performed to determine safety and immunogenicity of this vaccine.

Patients and Methods: Stage III to IV breast cancer patients received up to 15 (200 μg) doses of the vaccine by intramuscular injection. The first five doses (induction phase) were given at 2-week intervals, with the remaining treatment (maintenance) administered on a monthly basis.

Results: Twenty-one patients, 11 of whom had metastatic disease, were included. Main toxicities included erythema and induration at the injection site, sometimes associated with mild pain, and low-grade fever (World Health Organization grades 1 and 2). All treated patients who completed the induction phase developed anti-NeuGcGM3 antibody titers between 1:1,280 and 1:164,000 immunoglobulin G (IgG), and 1:640 and 1:164,000 IgM. Noteworthy specific IgA antibodies were induced by vaccination in all stage III patients and in three stage IV patients. Serum antibody levels were higher in the stage III patients, with the larger increases observed after week 32. The antiganglioside IgG subclasses were mainly IgG1 and IgG3. Hyperimmune sera increased complement-mediated cytotoxicity versus P3X63 myeloma cells and a marked IgG differential reactivity against human mammary ductal carcinoma samples.

Conclusion: NeuGcGM3/VSSP/Montanide ISA 51 is an unusual immunogenic ganglioside vaccine and also seems to be safe in this small trial. Immunologic surrogates of activity indicate that this reagent warrants further investigation.

J Clin Oncol 21:1015-1021.

CHANGES IN carbohydrate pattern expression associated with malignant transformation have been described for certain types of cancer.[1] In particular, overexpressed gangliosides have been considered attractive targets for immunotherapy[2,3] because either vaccination[4,5] or passive immunotherapy[6,7] of cancer patients with monoclonal antibodies against gangliosides has shown promising results. Nevertheless, recent clinical evidence[8] that GMK (ganglioside Memorial Kettering; a GM2 ganglioside vaccine) is inferior to interferon alfa for the treatment of high-risk melanoma patients has seriously hampered this concept. One possible explanation of this relative failure could be related to an insufficient enhancement of GM2 immunogenicity provided by this particular vaccine.

We speculated whether NeuGc-containing gangliosides could be better targets for cancer vaccines because of their predicted high immunogenicity, provided that Hanganutziu-Deicher (HD) gangliosides are present in human tumors. Though this has been a debatable subject,[9] some interesting observations are available: (1) anti-NeuGc containing ganglioside immune sera can recognize human tumors and cancerous cell lines,[10,11] (2) increased levels of NeuGcGM3 ganglioside are present in human breast tumors,[12] (3) three different monoclonal antibodies, 3.2E1[13] (anti-NeuGc STn mucin epitope), P3[14] (anti-NeuGc gangliosides), and 14F7[15] (anti-NeuGcGM3 ganglioside), reacted with breast cancer tissues by immunohistochemistry. These observations reinforced our proposition to study the use of NeuGcGM3 ganglioside as an attractive target for breast cancer immunotherapy.

In addition, we recently have shown that a vaccine, formulated by combination of gangliosides with the outer membrane protein complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP), increased the immunogenicity in mice of the tolerated gangliosides GM3 and NeuGcGM3.[16] VSSP technology allows the gangliosides to be placed in the context of potent Gram-negative innate immunity ligands and to keep them structurally intact.

This report describes, for the first time, the immunogenicity and toxicity profiles of the heterophilic ganglioside NeuGcGM3/VSSP/Montanide ISA 51 vaccine in advanced breast cancer patients.

PATIENTS AND METHODS

Patients

Patients were eligible for this study if they had histological proven stage III or IV breast cancer; were between 18 and 70 years of age; had appropriate marrow, liver, and renal function by conventional blood test; exhibited good performance status; and had no contraindication such as pregnancy, autoimmune disease, or active infections. Patients had to have recovered from the toxicity of any previous therapy for at least 4 weeks before trial entry.

A total of 21 advanced breast cancer patients were included in this study from the National Institute of Oncology and Radiobiology, Havana, Cuba.

Patients with progressive disease were excluded and treated with conventional therapies (radiotherapy or chemotherapy). Blood for serological studies were obtained on weeks 0, 2, 4, and 8 at the induction period and every 2 months until 1 year. All patients gave written informed consent to participate.

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Submitted February 25, 2002; accepted December 9, 2002.

Supported in part by Laboratorios ELEA (Buenos Aires, Argentina) and the Cuban Government.

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0732-183X/03/2106-1015/$20.00

Gangliosides

GM3 and NeuGcGM3 were obtained from dog and horse erythrocytes by a modification of the Folch method.[17]

NeuGcGM3/VSSP Vaccine

Vaccine lots were prepared as described by Estevez et al[16] in the good manufacturing process (GMP) facilities of the Center of Molecular Immunology (Havana, Cuba). All patients received 200 μg of NeuGcGM3/VSSP cancer vaccine with Montanide ISA 51 adjuvant (Seppic, Paris, France). The emulsified vaccine was injected intramuscularly on weeks 0, 2, 4, 6, and 8 at the first 2 months (induction period) and monthly until the first year (15 doses).

Serologic Procedures

Enzyme-linked immunosorbent assay (ELISA) experiments using gangliosides were carried out as previously described.[12] In brief, gangliosides (200 ng/well) in 50 μL of methanol were dried in 96-well polystyrene plates (PolySorp, Nunc, Denmark) for 90 minutes at 37°C. Then the plates were washed with phosphate-buffered saline containing Tween 20 (0.05% vol/vol). Serum samples dilutions were incubated overnight at room temperature. After samples were washed with phosphate-buffered saline containing Tween 20, biotinylated conjugated goat antihuman immunoglobulin M (IgM) or antihuman IgG (Jackson Immunoresearch Laboratories, Inc, West Grove, PA) was added and incubated for 1.5 hours at 37°C. After a new wash, streptavidin-alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories) was used in the same incubating conditions. The plates were washed again and a *p*-nitrophenylphosphate (Sigma Chemical Co, St Louis, MO) in diethanolamine buffer (pH 9.8) solution was added. After 30 minutes, absorbances were measured at 405 nm with an ELISA reader (Organon Teknika, Salzburg, Austria)

For IgG subclass determination, biotinylated monoclonal antibodies, specific for human IgG1, IgG2, IgG3, and IgG4 (PharMingen, San Diego, CA), were used with the same ELISA protocols previously described for gangliosides. Complement-dependent cytotoxicity (CDC)[18] assays were performed by a 2-hour ^{51}Cr release assay. Cells from the NeuGcGM3 positive myeloma cell line P3X63[19] served as target cells. Cells (3×10^6) were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Pharmacia Biotech, Buckinghamshire, UK) in 10% fetal calf serum RPMI for 2 hours at 37°C under 5% of CO_2. Cells were washed twice, and 10^4 cells/well in round-bottom 96-well plates (COSTAR, Cambridge, MA) were incubated with different serum concentrations for 4 hours at 37°C in the presence or absence of rabbit complement (1:5 and 1:10 dilutions). The plates were spun at $500 \times g$ for 5 minutes, and aliquots of 100 μL of supernatant of each well were harvested for determination of released ^{51}Cr. All assays were performed in triplicate and included control wells for maximum release in 5% sodium dodecyl sulfate (Sigma) and 5% Triton X-100 (Riedel de Haën, Seelze, Germany) and for spontaneous release in the absence of complement. The percentage of specific lysis was calculated as follows:

% Cytotoxicity = (experimental release − spontaneous release)/(maximum release − spontaneous release)

Indirect Immunoperoxidase Staining

For detection of breast cancer–reactive serum IgG, the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was employed according to the instructions of the manufacturer. Formalin-fixed tumor specimens, diagnosed as invasive mammary ductal carcinoma, were obtained from Evita Pueblo General Hospital (Berazategui, Argentina) and processed using the usual paraffin technique. Tumor sections (5 μm) were incubated for 1 hour with immunized or preimmunized patient sera at a dilution of 1:500 in phosphate-buffered saline. Slides were then incubated with biotinylated goat antihuman IgG for 30 minutes, followed by peroxidase-conjugated avidin-biotin complex for 30 minutes. Bound antibodies were detected by incubation with the peroxidase substrate diaminobenzidine for 2 to 4 minutes, and tumor sections were then counterstained with hematoxylin. Pertinent specificity tests were performed, including blocking of endogenous peroxidase, omission of the first serum, and use of preimmune serum.

Statistics

The statistical significance of comparisons in serum CDC values between stage III and IV patients were determined with the Mann-Whitney test.[20]

RESULTS

Patient Population

Twenty-one advanced breast cancer patients were studied for serologic response and toxicity symptoms. Patient characteristics are shown in Table 1. Eight patients completed the entire treatment schedule of 15 vaccine doses. Eleven patients were removed from the study because of disease progression, and another two patients refused to continue the trial.

Toxicity

The vaccine toxicity profile is listed in Table 2. The most frequent adverse effect was short-lasting pain in the injection site. Local skin reactions such as induration, erythema, tenderness, and swelling usually occurred at the injection site and disappeared in 1 to 3 days. Fever (grade 1 or 2, according to World Health Organization [WHO] criteria) that disappeared spontaneously or by usual antipyretic treatment was also observed. Grade I cephalea, myalgias, and arthralgias were re-

Table 1. Patient Characteristics

Variable	No. of Patients	%
No. of registered patients		
No. of eligible patients	21	
No. assessable for response	17	80.95
No. assessable for response (abandoned)	2	9.52
Female WHO performance status		
0	15	71.42
1	5	23.8
2	1	4.8
Age, years		
Median (N = 21)	59	
Range	36 to 70	
Histology		
Breast ductal carcinoma	15	71.42
Breast tubular carcinoma	1	4.8
Breast lobular carcinoma	1	4.8
Other breast carcinoma	4	19.04
Stage		
Locally advanced	10	47.61
IV	11	52.38
Organ involvement		
Breast only	10	47.61
One metastatic site	5	23.8
Two metastatic sites	5	23.8
Three metastatic sites	1	4.8
Prior therapy		
Surgery, radiotherapy, chemotherapy, and hormone therapy	10	47.61
Surgery, radiotherapy, and chemotherapy	3	14.28
Surgery, radiotherapy, and hormone therapy	1	4.8
Surgery, chemotherapy, and hormone therapy	2	9.52
Radiotherapy, chemotherapy, and hormone therapy	3	14.28
Chemotherapy, and hormone therapy	1	4.8
Nil	1	4.8
Concurrent hormone therapy		
Locally advanced	4	19.05
IV	5	24.71

Table 2. Adverse Events

Adverse Events	Grade I	Grade II
Pain in the injection site	21	
Local erythema	14	3
Induration in the injection site	3	
Fever	4	17
Cephalea	7	1
Chills	14	1
Nausea	1	1
Vomiting	3	2
Arthralgia	5	
Myalgia	2	
Increase of alkaline phosphatase	1	
Increase of creatinine	1	
Increase of AST	1	
Leukopenia	2	
Anemia	1	
Granulomas	1	
High blood pressure	2	
Decrease in blood pressure	1	

ported in some patients, although less frequently than fever, pain in the injection site, local erythema, and chills. Laboratory tests of four patients revealed anemia, leukopenia, and increase of creatinine and AST (all WHO grade 1). These adverse effects were not considered to be associated with vaccination. One patient developed a small abscess in the injection site that required a change in the injection site from arm to gluteus.

Anti-NeuGcGM3 Antibody Responses

The trial protocol established that only patients who received at least five vaccine doses were immunologically valuable. The pre- and postimmune antibody titers against NeuGcGM3 in the 15 selected patient's sera are shown in Tables 3 and 4. Either IgM or IgG antibodies (titers 1:80 to 1:640) were present in some sera before vaccine administration. Specific IgM and IgG were detected in 50% of sera from patients with stage III disease. Preimmnune dual presence of IgM and IgG antibodies was observed only in one patient. Conversely, the same frequencies of previous anti-NeuGcGM3 IgG antibodies and 33% for IgM were observed for stage IV patients as for stage III patients. Sera from two patients showed combined presence of both classes of antibodies.

After immunization, a high anti-NeuGcGM3 IgM antibody response was induced in all stage III patients (maximum titer range, 1:10,240 to 1:164,000), whereas only half of stage IV patients developed maximum titers greater than 1:10,000 (Table 3). High-titer IgG-specific antibodies were also induced in 90% of stage III evaluable patients (maximum titer range, 1:10,240 to 1:164,000), although IgG titers above 1:10,000 were only detected in 33% of sera from stage IV patients. It is noteworthy that this unusual anticarbohydrate class of immunoglobulin was induced by vaccination in all patients (titer range, 1:2,560 to 1:10,240; Table 4). For further characterization of this unusually high antiganglioside IgG response, a titer ratio was obtained by calculating the ratio between the highest observed titer after the induction phase of immunization and the titer value measured in week 8. This ratio allows the determination of an increase in antibody titers after the reimmunization schedule. A clear-cut stage-dependent difference was observed between obtained titer ratio values of 8 or more for eight of nine stage III patients and for two of six stage IV patients. Surprisingly, for most stage III patients, anti-IgG titers peaks were reached after receiving the tenth vaccine dose (Table 4). Anti-NeuGcGM3 IgG subclasses were studied in sera from eight patients. Predominant increases of IgG1 and IgG3 were observed, and only a few patients developed IgG2. No specific IgG4 was detected (data not shown).

The presence of IgA anti-NeuGcGM3 serum antibodies also was studied. No prevaccination IgA was detected in any patient (Table 5), whereas after immunization, all stage III patients developed this class of immunoglobulins (maximum titer range, 1:640 to 1:10,240). Conversely, only 50% of stage IV patients developed specific IgA antibodies (maximum titer range, 1:640 to 1:2,560), which lasted until the end of the treatment period.

Immunohistochemistry

The reactivity of serum IgG from patients immunized with the NeuGcGM3/VSSP vaccine was examined by a sensitive immunoperoxidase assay on human mammary ductal carcinoma specimens. The pre- and postimmune sera from four patients were

Table 3. IgM Titers Versus *N*-Glycolyl GM3 in Patients Who Received at Least Five Doses

Patient	Stage	No. of Doses	Weeks 0	8	16	24	32	40	48
6	III	15	0	2,560	1,280	320	1,280	20,480	20,480
8	III	15	ND	5,120	40,960	20,480	10,240	40,960	20,480
12	III	12	80	5,120	10,240	5,120	10,240		
15	III	15	160	20,480	20,480	163,840	81,920	5,120	5,120
17	III	15	0	2,560	2,560	1,280	5,120	2,560	10,240
18	III	15	0	10,240	5,120	10,240	10,240	2,560	2,560
19	III	15	80	2,560	5,120	ND	10,240	ND	20,480
20	III	11	0	2,560	5,120	40,960	20,480		
21	III	8	80	10,240	10,240	5,120			
1	IV	5	0	10,240					
3	IV	9	0	2,560	320	640			
4	IV	8	0	5,120	10,240				
9	IV	15	80	640	1,280	1,280	640	640	320
10	IV	8	0	1,280	5,120	2,560			
11	IV	15	640	40,960	40,960	20,480	2,560	1,280	320

Abbreviations: IgM, immunoglobulin M; ND, not determined.

Table 4. IgG Titers Versus *N*-Glycolyl GM3 in Patients Who Received at Least Five Doses

Patient	Stage	No. of Doses	Titer Ratio*	Weeks 0	8	16	24	32	40	48
6	III	15	32	80	2,560	2,560	1,280	2,560	*81,920*	81,920
8	III	15	16	ND	2,560	2,560	5,120	5,120	*40,960*	20,480
12	III	12	8	0	2,560	1,280	1,280	*20,480*		
15	III	15	32	0	1,280	10,240	5,120	20,480	5,120	*40,960*
17	III	15	8	0	640	2,560	2,560	5,120	2,560	*5,120*
18	III	15	2	160	5,120	5,120	5,120	*10,240*	2,560	2,560
19	III	15	512	80	320	5,120	ND	2,560	ND	*16,3840*
20	III	11	128	80	160	5,120	10,240	*20,240*		
21	III	8	32	0	2,560	*81,920*	10,240			
1	IV	5	†	80	10,240					
3	IV	9	0.25	0	5,120	320	1,280			
4	IV	8	4	0	640	2,560				
9	IV	15	8	80	320	*2,560*	1,280	640	1,280	2560
10	IV	8	8	0	1,280	*10,240*	5,120			
11	IV	15	2	640	2,560	320	2,560	2,560	5,120	1280

NOTE: Values in italics represent the higher titer after the induction phase.
Abbreviations: IgG, immunoglobulin G; ND, not determined.
*Titer ratio is ratio between the highest observed titer after the induction phase of immunization with respect, to the titer value measured in week 8.
†Titer ratio value cannot be determined.

evaluated, two of which showed a marked differential reactivity after the vaccination protocol. The pattern observed using the reactive sera showed that most carcinoma cells displayed a distinct strong positive staining, whereas surrounding connective tissue showed a mild, background reactivity (Fig 1). The other two sera displayed different behaviors. One of the sera samples showed high reactivity in preimmune and in postimmune conditions over the range of dilution used, and another sera sample did not show reactivity, even in the most concentrated dilution.

CDC

Tested patient's hyperimmune sera recognized the P3X63 myeloma cell line by fluorescence-activated cell sorter analysis (data not shown). Another interesting difference between the serological behavior of patients with stage III and IV breast cancer was observed in the preimmune sera CDC values, which are probably associated with xenogenic antibodies or the alternative pathway of complement activation. The median CDC preimmune value was 3.26% (range, 0.8% to 4.6%) for stage IV patients, whereas it was 23.5% (range, 5.2% to 42.4%) for stage III patients (P = .004, Mann-Whitney test). The same differences in serum CDC potency were observed between stage III and IV patients after vaccine administration (Table 6). Median CDC values for sera from stage III and IV patients were 82.4% (range, 69.9% to 97.4%) and 48.3% (range, 39.6% to 74.3%), respectively (P = .009, Mann-Whitney test). Nevertheless, a significant net increase in CDC potency was observed for all tested sera after vaccination, irrespective of the patient's disease stage.

DISCUSSION

For the first time, a heterophilic ganglioside vaccine was tested in advanced cancer patients. The primary goal of this phase I clinical trial with the NeuGcGM3/VSSP/Montanide ISA 51 vaccine was to study its toxicity and immunogenicity in the selected vaccination regimen. The vaccine's safety was demon-

Table 5. IgA Titers Versus *N*-Glycolyl GM3 in Patients Who Received at Least Five Doses

Patient	Stage	No. of Doses	Weeks 0	8	16	24	32	40	48
6	III	15	0	640	640	640	1,280	2,560	2,560
8	III	15	ND	5,120	10,240	10,240	5,120	5,120	2,560
12	III	12	0	1,280	1,280	640			
15	III	15	0	640	2,560	160	160	1,280	640
17	III	15	0	0	0	80	160	640	640
18	III	15	0	640	2,560	2,560	1,280	2,560	640
19	III	15	0	10,240	10,240	ND	10,240	ND	10,240
20	III	11	0	2,560	1,280	ND	320		
21	III	8	0	640	1,280	640			
1	IV	5	0	0					
3	IV	9	0	0	0	0			
4	IV	8	0	2,560	0				
9	IV	15	0	160	320	1,280	1,280	640	640
10	IV	8	0	640	2,560	1,280			
11	IV	15	0	320	0	640	320	640	640

Abbreviations: IgA, immunoglobulin A; ND, not determined.

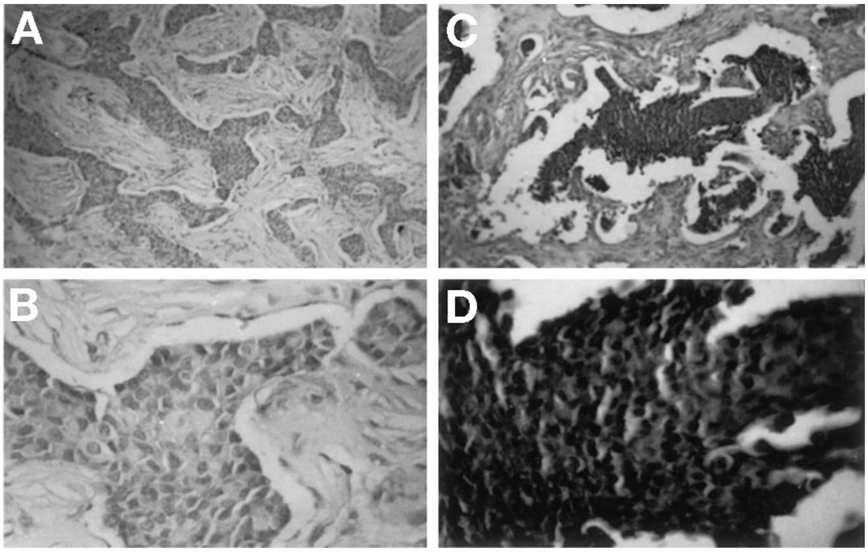

Fig 1. Immunochemistry using sera from patients immunized with NGcGM3/VSSP vaccine. Ductal mammary carcinoma sections were incubated with preimmune ([(A) ×100 and (B) ×400] and postimmune sera [(C) ×100 and (D) ×400] and revealed with the peroxidase diaminobenzidine substrate. Optimal serum dilution (1:500) is shown.

strated conclusively. No evidence of serious or unexpected adverse effects was observed after a 1-year period of administration. In contrast to another ganglioside vaccine (GMK),[21] no grade 3 or 4 toxic events, according to WHO criteria, were reported. It is a curious finding that between 33% and 50% of sera from advanced breast cancer patients showed anti-NeuGcGM3 antibody titers before vaccination. In melanoma patients, a correlation between high anti-*N*-glycolylated gangliosides IgM and IgG antibody titers and disease-free intervals, 5 years after surgery, has been reported.[22] Whether the presence of these natural antibodies could reflect a reaction of the immune system against this tumor-specific antigen remains unclear. An increase in anti-NeuGcGM3 IgM and IgG antibody titers was seen in all patients immunized with at least five doses of the NeuGcGM3/VSSP/Montanide ISA 51 cancer vaccine. The frequency of high responders (maximum titers > 1:10,000) depended on the stage of the disease and was much higher for stage III patients. The observed specific antibody titer peak values for either IgM or IgG (induced by this vaccine administration protocol) are unprecedented, probably because of the heterophilic characteristic of NeuGcGM3 in humans and the peculiar immunogenic properties of the VSSP approach.

Table 6. Vaccinated Patients Serum Complement–Mediated Cytotoxicity

Patient	Stage	Cytotoxicity (%) Prevaccination	Cytotoxicity (%) Postvaccination
6	III	25.3	69.9
12	III	5.2	97.4
15	III	21.2	90.7
17	III	42.4	78.0
18	III	17.1	85.0
19	III	29.8	79.8
3	IV	3.9	74.3
4	IV	0.8	39.6
9	IV	4.4	48.3
10	IV	2.6	66.7
11	IV	4.6	48.3

The value of vaccine-induced antiganglioside antibody titers for cancer therapy remains a polemic matter. A better prognosis was found in melanoma patients with anti-NeuGcGM3 IgG serum antibodies after the immunization with a vaccinia virus human melanoma cells oncolysate.[23] More recently, Takahashi et al[24] reported that the levels of antiganglioside IgM antibodies against GD2, GM2, GD3, and GM3 correlated with survival in melanoma patients who had undergone resection of regional metastases (American Joint Committee on Cancer stage III) followed by adjuvant immunotherapy with CancerVax (a whole melanoma cells vaccine). These results, together with the outcome of the classical report by Livingston et al[25] in which stage III melanoma patients developing natural antibodies against GM2 or induced by a GM2/bacillus Calmette-Guérin vaccine showed an increase in the disease-free interval and the overall survival, indicate the importance of antiganglioside antibodies in the outcome of melanoma. Nevertheless, a recent phase III trial in which high-dose interferon alfa-2b demonstrated a significant treatment benefit over GMK vaccine in melanoma patients has hampered this concept.

One possibility of ganglioside vaccine optimization is to achieve a more T-cell–dependent pattern of immune response. Two of the main characteristics of this type of response, high titer of IgG and the booster effect, were both observed in our vaccinated stage III breast cancer patients. Note that 90% of these patients showed a more than eight-fold increase in serum IgG titers compared with the primary response. Moreover, vaccination with the NeuGcGM$_3$/VSSP/Montanide ISA 51 vaccine induced a Th1-like pattern of specific IgG subclass distribution. Most stage III and IV patients predominantly showed antiganglioside IgG1 and IgG3 subtypes. These data are in accordance with another report related to a different vaccine formulation containing GM2 ganglioside.[3]

The finding of serum IgA antibodies against NeuGcGM3 in all stage III and 50% of stage IV patients was unexpected. To our knowledge, this is the first time that serum IgA antibodies against gangliosides have been detected after immunization with a ganglioside vaccine. IgA response against gangliosides previously has been reported only in patients who developed autoimmune diseases generally associated with intestinal disorders[26] and could be therapeutically useful in malignancies of the breast, intestine, or respiratory tract (the IgA role is a first line of mucosal defense).[27] This particular switch to secretory immunoglobulins could be associated with the VSSP components derived from *Neisseria meningitidis*, as has been described.[28]

The CDC study showed a potent cytotoxic effect in all sera of selected patients after vaccination, independent of their stage. Interestingly, Hsueh et al[29] reported a significant correlation between disease-free survival and serum CDC against melanoma cells in stage III patients vaccinated with Canvaxin after surgical resection of regional node metastases (adjuvant setting). We also found that this cytotoxic effect was significantly higher for sera from stage III patients compared with the correspondent more advanced patients, reinforcing the observed fact that the serological potential of vaccination diminishes with disease progression.

In addition, a strong positive staining against paraffin-embedded human mammary carcinoma tissue sections using sera from selected patients immunized with the NeuGcGM3/VSSP vaccine was found. A question exists whether the solvents used in paraffin dehydration and inclusion of the tissue could remove an important part of cell surface lipids. Recently, we demonstrated a strong positive staining using GM3-immunized sera against paraffin embedding GM3-expressing melanoma tumors. In this work, we assayed different resins instead of paraffin, such as Epon and Spur (Electron Microscopy Sciences, Fort Washington, PA), which minimize the lipid extraction of the tissue, and showed that the same reactivity is reached in all cases.[30]

The strong serum reactivity against mammary carcinoma cells demonstrated the potent humoral immune response achieved by the NeuGcGM3/VSSP vaccination. These results indicate one of the possible antitumoral mechanisms of action in which antibody recognition of tumor cells may be involved.

Although a phase I trial is not adequate for efficacy assessment, two patients with pulmonary metastases remained stable 18 and 40 months, respectively, after entry in the trial.

In summary, we might reasonably conclude that the NeuGcGM3/VSSP/Montanide ISA 51 vaccine was immunogenic and safe on advanced breast cancer patients, taking into account the limited number of included patients. Furthermore, the vaccine administration in the adjuvant setting may be more effective because of the observed higher immunogenicity induced in stage III patients. In addition, vaccination schedules including prolonged periods of reimmunizations with the NeuGcGM3/VSSP/Montanide ISA 51 preparation may be more adequate.

ACKNOWLEDGMENT

The acknowledgment is available online at www.jco.org.

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