A Phase I Study of an Allogeneic Cell Vaccine (VACCIMEL) With GM-CSF in Melanoma Patients

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Summary: We investigated whether recombinant human granulocyte-monocyte-colony-stimulating factor (rhGM-CSF) increased the immunogenicity of VACCIMEL, a vaccine consisting of 3 irradiated allogeneic melanoma cell lines. A phase I clinical trial was performed on 20 melanoma patients in stages IIB (n = 2), III (n = 10), and IV (n = 8), who were disease free after surgery (n = 16) or had minimal disease (n = 4). Cohorts of 4 patients were vaccinated 4 times with VACCIMEL and bacillus Calmette Guerin (BCG) as adjuvant. Besides, the patients received placebo (group 1) or GM-CSF: 150 μg (group 2), 300 μg (group 3), 400 μg (group 4), and 600 μg (group 5) per vaccine. The combination of VACCIMEL and GM-CSF had low toxicity. Only in group 5, grade 2 thoracic pain (3/4 patients) and abdominal cramps (2/4 patients) were observed. Delayed-type hypersensitivity increased after vaccination and it was highest in group 4. Phytohemagglutinin stimulation of peripheral blood lymphocytes was analyzed in 9 patients: 4/9 had normal stimulation; 3/9 had low basal stimulation, which recovered after vaccination; and 2/9 were not stimulated. Antimelanoma antibodies preexisted in 9/19 patients; in 3/19 patients, antibodies anti-33 kd, 90 kd, and 100 kd antigens were induced by vaccination. IgG2 but not IgG1 antibodies were detected. Anti-BCG antibodies, mostly IgG2, reached the highest post/prevaccination ratio in group 4. Median serum interleukin-12 was lower in progressing patients (61.6 pg/mL) than in those without evident disease (89 pg/mL). Thus, its low toxicity and the induction of a predominantly cellular immune response suggest that the addition of 300 to 400 μg GM-CSF to VACCIMEL is useful in increasing the immune response.

Received for publication July 19, 2005; accepted January 2, 2006.

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Supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), the University of Buenos Aires, the Fundación Sales, the Fundación para la Investigación y Prevención del Cancer (FUCA), the Fundación Pedro F. Mosoteguy, and the Fundación María Calderón de la Barca, Argentina.

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Key Words: melanoma vaccine, GM-CSF, BCG, clinical trial

(*J Immunother* 2006;29:444–454)

Malignant melanoma is the cancer with the fastest growing incidence, and the survival of patients with metastatic melanoma is often less than 1 year.[1] The use of chemotherapy in this disease is quite deceiving, because mostly short-lived partial responses are obtained. In addition to this, Lev et al[2] recently reported in experimental systems that treatment with dacarbazine could select a more aggressive tumor cell phenotype. Therefore, the use of therapeutic vaccines has attracted considerable interest in recent years. Among the different available vaccination strategies, the use of irradiated allogeneic tumor cells is based on the paradigm that tumor cells would only trigger MHC-restricted tumor-specific immunity after being phagocytosed by dendritic cells (DCs), the main initiators of immune response[3] and which exist basically in immature and mature states. In C57/BL6 mice, we have shown that immature DCs phagocytose apoptotic B16 melanoma cells and induce long-lived protective immunity, $CD4^+$ and $CD8^+$ T-cell dependent, against B16 melanoma.[4] After phagocytosis, DCs evolve to a mature phenotype, diminish their phagocytic ability, express human leukocyte antigen (HLA) II and costimulatory molecules on their surface, and acquire the capacity to process tumor antigens and present them in the adequate self-HLA context. Coincidently, and through the expression of adequate chemokine receptors, such as CCR7,[5] DCs are able to travel to the draining lymph nodes to prime naive lymphocytes and trigger cellular and/or humoral immunity.[6] The correct presentation of tumor antigens to the patient's lymphocytes would depend therefore on the DCs, but not on the vaccine, haplotype. In this scenario, it is crucial to increase the physical contact between tumor cells and DCs. There is abundant evidence that GM-CSF is a powerful chemoattractant of monocytes and that it induces their maturation into DCs. It has been shown in experimental systems[7] and in clinical trials, using retroviral[8] or adenoviral vectors,[9] that irradiated autologous tumor cells transfected with the GM-CSF gene attain cytokine concentrations able to induce protection against challenge in animal models, and stimulation of immunity in melanoma patients. In these vaccination clinical trials,

the accumulation of large numbers of DCs, macrophages, eosinophils, neutrophils, and T lymphocytes at the vaccination site suggests that GM-CSF augments tumor antigen presentation.[8,9] An alternative approach to GM-CSF gene transfection is to perform local injections of GM-CSF. Thus, Kremer et al[10] demonstrated that local injection of GM-CSF induced an afflux of CD1a (+) S100 (+) Langerhans cells from the epidermis to the dermis, and that local immunization was more efficient after local treatment with the cytokine. Also, Nasi et al[11] and Hoeller et al[12] injected GM-CSF i.d. in patients with cutaneous melanoma metastases, and observed an increase in DCs and regression of some metastases. It has also been reported by Leong et al[13] that vaccination in melanoma patients with autologous tumor cells plus soluble GM-CSF induced responses in 20% of the cases. We have previously developed an allogeneic melanoma vaccine (VACCIMEL), which has been assayed in a phase II clinical trial on 30 melanoma patients and which increased the disease-free survival from 7.0 months in a historical control group to 20.0 months in the vaccinated group ($P < 0.001$).[14] With a follow-up range between 47 and 88 months, 9 patients (30%) are still disease free (Mordoh et al, to be reported elsewhere). To investigate whether the addition of GM-CSF to VACCIMEL increases the immune response to tumor antigens, and the toxicity of this association, we performed a phase I study on 20 melanoma patients. Several immunologic parameters were determined in this study: (i) delayed-type hypersensitivity (DTH), as it has been demonstrated that skin tests predict survival in melanoma patients[15]; (ii) the presence of serum antitumor antibodies; (iii) as the patients were not selected on the basis of HLA haplotype, polyclonal T-cell response to the nonspecific stimulant phytohemagglutinin (PHA) of prevaccination and post-vaccination peripheral blood lymphocytes was measured. This response has been found to detect a compromise of immune function[16,17]; (iv) the serum levels of interleukin-10 (IL-10) and interleukin-12 (IL-12), anti-inflammatory and proinflammatory cytokines,[18] respectively, were also measured. The augmentation of serum levels of IL-10 has been associated with the worst prognosis in patients with hematologic and solid tumors, including melanoma.[19–22] With respect to the proinflammatory cytokine IL-12, there are no data to our knowledge correlating serum levels of IL-12 with cancer extent or prognosis. We decided therefore to analyze in our group of patients the presence of IL-10 and IL-12, both before and after vaccination. We report in this study for the first time that serum IL-12 levels are lower in melanoma patients with unfavorable clinical evolution.

MATERIALS AND METHODS

VACCIMEL

Three human melanoma cell lines comprise the vaccine: IIB-MEL-J, IIB-MEL-LES, and IIB-MEL-IAN, which were cultured in melanoma medium as previously described.[23,24] The cell lines were grown in a GMP core facility at the Centro de Investigaciones Oncológicas—FUCA. After irradiation at 50 Gy (Siemens Lineal Accelerator), the cells were frozen (50% Dulbecco Modified Eagle Medium, 40% human albumin, and 10% dimethyl sulfoxide) in liquid nitrogen until use. For each vaccine, 5×10^6 cells of each cell line were thawed, washed, mixed, and resuspended in Dulbecco Modified Eagle Medium. Bacillus Calmette Guerin (BCG) was from the Instituto Nacional de Microbiología Carlos Malbrán, Buenos Aires, Argentina. Nonglycosylated recombinant human GM-CSF (rhGM-CSF; Molgramostim) was a generous gift from Dr Esteban Corley, PC Gene, Buenos Aires, Argentina.

Patients

This phase I study received approval from the Institutional Review Board of the Instituto Alexander Fleming, from an independent Ethics Committee, and from the ANMAT, Ministry of Health (Argentina). The patients were eligible for enrollment under the following conditions: (a) cutaneous melanoma, histologically confirmed; (b) age between 15 and 70 years; (c) life expectancy > 6 months; (d) performance status (Eastern Cooperation Oncology Group) 0 or 1; (e) clinical stages IIB, III, and IV (American Joint Committee on Cancer). Stage IV patients had minimal or nondetectable disease after surgery as asserted by CAT scans and lactate dehydrogenase values; (f) patients with stage III disease had to be previously treated with interferon (IFN)-α, and either finished the treatment or suspended it owing to disease progression or toxicity; (g) laboratory eligibility criteria included: hematocrit > 35%; white blood cell count > 3500/mm^3, platelets > 100,000/mm^3; total and direct bilirubin, serum oxalacetic transaminase, and glutamic pyruvic transaminase < 1.5-fold the upper normal value; lactate dehydrogenase ≤ 450 mU/mL; (h) absence of pregnancy, with serum β-human chorionic gonadotrophin determined 1 week before each vaccination in premenopausal women; (i) no chemotherapy, radiotherapy, or biologic treatment during the previous month; (j) no concurrent medication with corticosteroids or nonsteroidal anti-inflammatory drugs; (k) no active brain metastases; and (l) all patients gave informed consent.

Vaccination

The vaccine was injected i.d. in one of the extremities with intact draining lymph nodes. Group 1 patients were given VACCIMEL plus placebo. Recombinant human granulocyte-monocyte-colony-stimulating factor (rhGM-CSF) doses were as follows: group 2: 150 μg (37.5 μg/d, 4 d); group 3: 300 μg (75 μg/d, 4 d); group 4: 400 μg (100 μg/d, 4 d); and group 5: 600 μg (150 μg/d, 4 d). On the vaccination day, 0.1 mL of placebo or the corresponding rhGM-CSF dose was gently mixed with VACCIMEL (15×10^6 irradiated cells in 0.3 mL) and BCG (2×10^6 colony-forming units in 0.05 mL). During the next 3 days, 0.1 mL of placebo or rhGM-CSF was injected ID at the vaccination site where a greater

induration was noticed. Every patient received 4 vaccinations 3 weeks apart. The patients were clinically examined 1 week before the start of vaccination, the 4 days of each vaccination, and 3 weeks after the last vaccination. At the 12th week, the patients' statuses were investigated with abdominal ecography and chest x-rays. Analysis of blood chemistry and differential white blood cell counts were performed 1 week before vaccination, the same day of the fourth vaccination, and at the 12th week to monitor systemic toxicity.

DTH Measurement

On each vaccination day, DTH was performed in the forearm with 1/10th of the VACCIMEL dose, without BCG or rhGM-CSF, and the reaction was read at 2, 24, 48, 72 hours, and afterward until the reaction faded (at least 4 readings). A DTH intensity value was established as follows: 0: erythema < 0.5 cm; 1: macular erythema 0.5 to 1.0 cm; 2: macular erythema 1.0 to 2.0 cm; 3: macular erythema > 2.0 cm; 4: papular erythema > 2.0 cm. A DTH score was defined that corresponds to the average of the sum of the DTH values recorded at the last 3 vaccinations. The DTH measured at vaccination no. 1 was not considered for statistics because it was always 0. Therefore, DTH score = Σ individual DTH intensities (at least 12 readings)/3. DTH measurements were not blinded.

Western Blots

VACCIMEL

Protein extracts were prepared from the three cell lines that comprise VACCIMEL. Cell pellets frozen at -80°C were thawed and treated during 20 minutes at 4°C with lysis buffer (50 mM Tris-ClH pH 7.5, 1% NP40, 150 mM NaCl, 5 mM ethylenediaminetetraaacetic acid, and 1 mM phenylmethlysulfonyl fluoride). The suspension was afterward homogenized with a Polytron (Brinkmann Instruments) and centrifuged for 40 minutes at 10,000*g*. The supernatant was aliquoted and frozen at -20°C. The protein concentration was measured according to Lowry.[25] Protein extracts were prepared equally from the human breast cancer cell line IIB-BR-G[26] and used to assess specificity in the Western blots.

Protein extracts (150 µg) were run in a 3% to 12% gradient SDS-PAGE and transferred to a nitrocellulose membrane (0.45 µm pore, Sigma-Aldrich, Saint Louis, MO). After blocking with 3% bovine skim milk (Moliko, Argentina), blots were incubated overnight at 4°C with 1/10 diluted patient's sera. After several washings, blots were either incubated with alkaline phosphatase-conjugated rabbit Ig antihuman IgG (DakoCytomation, Glostrup, Denmark), monoclonal anti-IgG1 or anti-IgG2 Fc-fragment (Calbiochem, San Diego, CA) and revealed with NBT/BCIP (Gibco, Carlsbad, CA). Identical blots were also incubated with an HRP-conjugated goat antihuman IgG + A + M (Zymed, San Francisco, CA) and revealed with 4-Cl-naphtol plus H_2O_2. As a control for secondary antibodies, a serum sample was incubated only with secondary antibody.

BCG

BCG was produced at the Instituto Malbrán (Buenos Aires, Argentina) at 120 mg/mL protein concentration. Bacilli were sonicated, aliquoted, and frozen at -20°C. After centrifugation to eliminate insoluble material, the supernatant was cracked in sample buffer, run in a 3% to 12% gradient SDS-PAGE, blotted, and incubated as described above with patient's prevaccination and postvaccination sera. For titration, sera were diluted 1/10, 1/100, 1/1000, and 1/5000; only postvaccination sera were diluted 1/10,000. Afterward, blots were incubated with HRP-conjugated goat antihuman IgG + A + M (Zymed, San Francisco, CA) and revealed as above. Alternatively, anti-IgG1 or anti-IgG2 were used as secondary antibodies.

rhGM-CSF

Two micrograms of rhGM-CSF (PC-Gen, Argentina) were run in a 12% SDS-PAGE, blotted to nitrocellulose membrane, incubated with prevaccination and postvaccination sera, diluted 1/10, and then with alkaline phosphatase-conjugated rabbit antihuman IgG polyclonal antibody (DakoCytomation, Glostrup, DK) and developed as described above. As a control, a serum sample was incubated only with secondary antibody.

Enzyme-linked Immunosorbent Assay (ELISA) for Cytokines in Serum

IL-10 and IL-12 concentrations were determined in the sera of vaccinated patients before (prevaccination serum) and 3 weeks after the fourth vaccination (postvaccination serum). Sera were frozen at -80°C until tested by ELISA (OptEIA IL-10 and IL-12, BD Biosciences, San Diego, CA) in triplicate. A calibration curve was performed for each experiment and the sample concentration was calculated by log-log regression analysis using Cembal 2.2 software.

Indirect Immunofluorescence of VACCIMEL with Prevaccination and Postvaccination Sera

Live melanoma cells that comprise VACCIMEL, mixed in equal proportions, were blocked with 10% rabbit serum for 30 minutes and incubated with 1/10 diluted prevaccination and postvaccination sera. After washing, the cells were incubated with rabbit antihuman IgG + A + M—FITC (DakoCytomation, Glostrup, DK) for 1 hour at 4°C. The cells were washed, fixed in 3% paraformaldehyde, and analyzed by FACS (FACSCalibur, BD, San Diego, CA). Alternatively, the cells were first permeabilized with 0.05% saponin (Sigma-Aldrich, Saint Louis, MO) in PBS in the blocking step and 0.05% saponin/PBS was added at each further step of the reaction. Normal donor serum as primary antibody was used as a control. For those patients whose postvaccination sera showed $> 15\%$ differences in the percentage of positive cells with respect to prevaccination sera, the immunofluorescence was repeated after 2 hours incubation with an excess anti-human HLA-ABC antigen (W6/32 clone, DakoCytomation, Glostrup, DK) to block

anti-HLA reactivity in the sera. A total of 10,000 events for each determination were analyzed.

Lymphocyte Stimulation With PHA

Prevaccination and postvaccination peripheral blood mononuclear cells were obtained by Ficoll-Hypaque density gradient purification and kept frozen in liquid nitrogen until use. For the assay, the cells were thawed and incubated in AIM-V medium (Gibco, Grand Island, NY) for 1 hour at 37°C. A total of 5×10^5 cells were plated in 96-well multiplates in the presence or absence of PHA (5 μg/mL) (Gibco, Grand Island, NY) and incubated at 37°C for 72 hours. During the last 16 hours, the cells were pulsed with (^{3}H)dThd (Amersham, 1 μCi/well) and radioactivity incorporated to DNA was measured after cell lysis (Cell Harvester, Nunc, Rochester, NY). The experiments were performed in triplicate and the mean ± SD are shown.

Histopathologic Analysis of Melanoma Metastases

Paraffin-embedded blocks of biopsies were used to analyze the lymphoid and DCs infiltrates. Sections of 3 to 5 μm thickness were stained with hematoxylin/eosin or immunostained with anti-CD4 (1F6 clone), anti-CD8 (C8E-144b clone), anti-CD20 (L26 clone), anti-CD1a (010 clone) (DakoCytomation, Glostrup, DK), and anti-CD57 (NK1 clone, Zymed, San Francisco, CA) antibodies and developed with ABC reagent and diaminobenzidine or Novared as substrates (Vectastain, Vector, Burlingame, CA). As a control, the reactions were performed omitting the primary antibodies. The sections were analyzed under an Olympus BX40 microscope.

Statistical Analysis

Because most data groups were not normally distributed, all data were analyzed by the Wilcoxon rank sum test. DTH scores of the different groups receiving rhGM-CSF were compared with group 1 (Placebo) values by 1-way analysis of variance and Dunnett multiple comparison test. $P < 0.05$ was considered significant.

RESULTS

Patients

This phase 1 study was performed on 20 melanoma patients who were recruited at the Instituto Alexander Fleming between October 2002 and January 2004. The characteristics of the patients are listed in Table 1. The age range was 15 to 67 years. There were 11 females and 9 males. Two patients were in stage IIB of the disease (AJCC); 10 patients in stage III, and 8 patients in stage IV. Patients PB and CS had subcutaneous metastases; patients ASS, SR, JV, and MB had surgery from lung metastases; GQ had surgery and irradiation from brain metastases; and JS had gastrointestinal metastases. Only 2 of the stage III patients had completed high-dose IFN-α treatment.[27] Cohorts of 4 patients were recruited. All the patients received one dose of VACCIMEL (0.3 mL) mixed just before vaccination with BCG and placebo or hrGM-CSF every 3 weeks, as described under Methods. Patient no. 6 was withdrawn from the protocol after the first vaccination due to rapidly progressing disease; this patient was not replaced.

Clinical Evolution of the Patients

As of October 2005, with a mean follow-up of 27.8 months (21 to 35 mo), the 2 stage IIB patients show nonevident disease (NED); 7/10 (70%) stage III patients

TABLE 1. Characteristics of the Patients Participating in the Clinical Trial

Patient	Sex	Age	Clinical Stage	Mts	Treatment Group	Clin. Evolution
1. M.G.	F	33	III	ND	Placebo	NED (35 m+)
2. P.B.	M	27	IV	ND	Placebo	D
3. M.R.C.	F	49	III	ND	Placebo	NED (34 m+)
4. A.S.S.	F	54	IV	ND	Placebo	D
5. S.R.	M	32	IV	ND	GM-CSF 150 μg	D
6. C.S.	F	27	IV	SC	GM-CSF 150 μg	WP-D
7. M.B.	F	32	III	ND	GM-CSF 150 μg	NED (32 m+)
8. G.Z.	M	33	IIB	ND	GM-CSF 150 μg	NED (30 m+)
9. J.V.	M	67	IV	Lung	GM-CSF 300 μg	D
10. G.Q.	F	36	IV	ND	GM-CSF 300 μg	NED (28 m+)
11. M.F.	F	48	III	ND	GM-CSF 300 μg	NED (27 m+)
12. M.A.F.	F	15	III	ND	GM-CSF 300 μg	NED (27 m+)
13. J.S.	M	37	IV	GI	GM-CSF 400 μg	D
14. A.F.A.	M	28	III	ND	GM-CSF 400 μg	P (3 m)
15. M.B.	M	25	IV	ND	GM-CSF 400 μg	D
16. R.B.	M	61	III	ND	GM-CSF 400 μg	P (5 m)
17. E.D.	F	61	III	ND	GM-CSF 600 μg	NED (23 m+)
18. H.I.	F	67	III	SC	GM-CSF 600 μg	P (4)
19. N.B.	M	45	IIB	ND	GM-CSF 600 μg	NED (21 m+)
20. S.V.N.	F	56	III	ND	GM-CSF 600 μg	NED (21 m+)

D indicates deceased; GI, gastrointestinal; m, months of follow-up; ND, nondetectable disease after surgery; P, progression; SC, subcutaneous; WP, withdrawn from protocol.

TABLE 2. Toxicity Associated With VACCIMEL+GM-CSF Treatment

Symptoms	VACCIMEL + Placebo	VACCIMEL + 150 μg GM-CSF	VACCIMEL + 300 μg GM-CSF	VACCIMEL + 400 μg GM-CSF	VACCIMEL + 600 μg GM-CSF
Fever	1/4	1/3	0/4	2/4	2/4
Fatigue	2/4	1/3	1/4	2/4	3/4
Headache	1/4	0/3	2/4	2/4	1/4
Tachycardia	1/4	0/3	0/4	0/4	0/4
Hypotension	0/4	0/3	0/4	1/4	1/4
Chills	0/4	0/3	1/4	0/4	1/4
Abdominal cramps	1/4	0/3	1/4	0/4	2/4
Thoracic pain	0/4	1/3	0/4	1/4	3/4
Palpitations	0/4	0/3	1/4	0/4	0/4
Local reaction	4/4	3/3	4/4	4/4	4/4
Myalgia	0/4	2/3	3/4	2/4	2/4

Toxicity was grade 1 in all cases except for local reaction, abdominal cramps, and thoracic pain, which were grade 2.

are NED and 3/10 patients experienced disease progression; and 1/8 stage IV patients are still NED (Table 1). No regression of metastatic lesions was observed in this study. Patient GZ (stage IIB) developed a low-grade pilocytic astrocytoma of the optic quiasma in July 2005, 24 months after completion of the study.

Toxicity

Local

The combination of VACCIMEL and BCG induced grade 2 erythema and induration at the vaccine site in all patients, which was excessive and led to a reduction in the BCG dose in 5/20 patients [PB (group 1), SR (group 2), MB and AFA (group 4), and NB (group 5)]. In these patients, the dose of BCG was diminished to one-fourth to avoid excessive local toxicity.

Systemic

The systemic toxicity of VACCIMEL, BCG, and rhGM-CSF was mild and no patient had to interrupt the treatment (Table 2). The most frequent toxicities were grade 1 fatigue (9/20) and grade 1 myalgias (9/20), followed by grade 1 fever (5/20). Grade 1 tachycardia (1/20) was observed in 1 patient of group 1. In group 5, 2/4 patients had moderate abdominal cramps and 3/4 patients had thoracic pain that subsided a few minutes after administration of 500 mg paracetamol when they were in a supine position.

The number of neutrophils and eosinophils increased considerably at rhGM-CSF doses higher than 300 μg. At the 600 μg dose, the mean increase in circulating neutrophils was > 50% and in eosinophils > 100%, clearly indicating that besides its local effect, rhGM-CSF was mobilizing granulocytes at the systemic level (data not shown).

Immune Response

DTH

Several parameters were used to analyze the immune response. DTH was measured at each vaccination, and the intensity of the reaction and the DTH score were determined as described under Methods (Fig. 1). The average DTH scores for each group were (mean ± SD): group 1: 3.2 ± 1.6; group 2: 1.9 ± 0.48; group 3: 3.5 ± 2.4; group 4: 10.0 ± 6.35; and group 5: 6.3 ± 2.4. The group 4 DTH score was statistically different from the placebo (group I) score ($P < 0.05$, Dunnett's multiple comparison test).

Antibodies Anti-VACCIMEL and Anti-BCG

To investigate whether addition of rhGM-CSF increased the humoral reactivity against tumor cells, we tested by FACS analysis the patient's sera reactivity against live VACCIMEL cells. It is interesting to note that almost every patient had some prevaccination reactivity. The number of patients who after vaccination displayed an increase higher than 15% in the percentage of positive cells was: 0/4 (group 1), 2/3 (group 2), 2/4 (group 3), 3/4 (group 4), and 1/4 (group 5). Similar results were observed after permeabilization of VACCIMEL cells. Some examples are shown in Figure 2. This reactivity was not abolished by pretreatment of the cells with a saturating concentration of anti-HLA-A,B,C antibody (not shown).

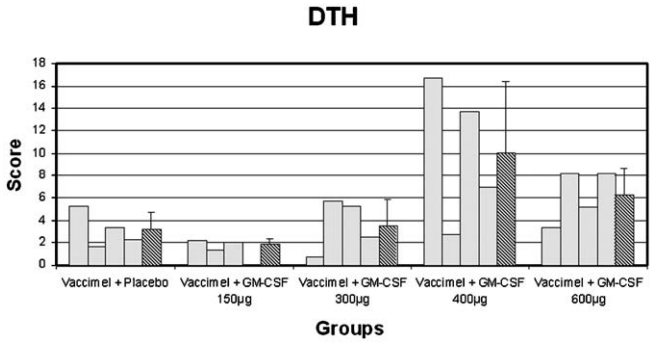

FIGURE 1. DTH score in vaccinated patients. The DTH score was calculated as described under Methods. Each patient's score is shown in gray bars and the mean ± SD of the vaccination group in striped bars.

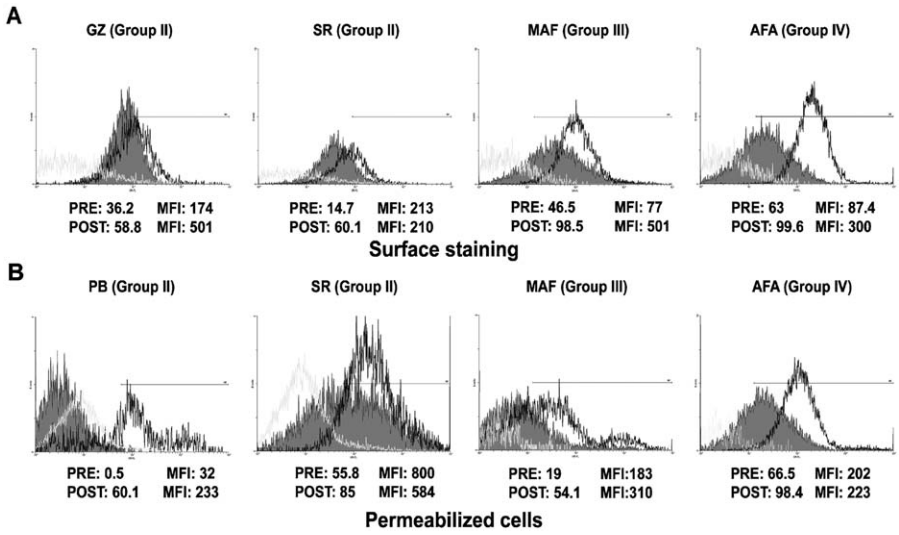

FIGURE 2. Humoral immune response measured by FACS. Serum reactivity of patients G.Z., S.R., M.A.F., A.F.A., and P.B. against live VACCIMEL cells was analyzed as described under Methods. A, Surface staining, B, permeabilized cells. The light gray line depicts normal serum staining; prevaccination serum staining is shown as a solid full line and postvaccination staining as a dark line.

We then tested the patient's sera (1/10 dilution) by Western blots against cell extracts of VACCIMEL. Extracts of the IIB-BR-G breast cancer cell line were used as a control of tumor specificity. Only 3/19 patients developed specific antibodies against 100 and 90 kd antigens after vaccination. As an example, the results obtained with patient GZ are shown (Fig. 3A). When the Western blots were developed with an IgG + IgA + IgM antibody, 100 and 90 kd antigens were detected in postvaccination but not in prevaccination serum (compare Fig. 3A, left panel). To investigate whether the anti-VACCIMEL antibody response induced by the vaccine corresponded to a T_H1 or T_H2 response, we investigated the presence of IgG1 and IgG2 antibodies in the patient's sera before and after vaccination, as it has been reported in humans that IgG2 production is related to T_H1 cytokines such as IFN-γ, whereas IgG1, IgG3, and IgG4 are rather related to T_H2 cytokines.[28]

When the Western blots of patient GZ were developed with anti-IgG1 or anti-IgG2 antibodies, no reactivity was detected with anti-IgG1 (Fig. 3A, center panel), but a melanoma-specific 33 kd antigen was detected in the sera before and after vaccination when anti-IgG2 was used. A 40 kd antigen was detected in VACCIMEL and IIB-BR-G extracts. In patient MAF, a similar pattern was observed: lack of reactivity with anti-IgG1 antibody (Fig. 3B, left panel) and reactivity against a 45 kd antigen, present both in prevaccination and postvaccination sera (Fig. 3B, center panel). In this case, no cross-reactivity against IIB-BR-G was detected. Nine out of 19 patients had antibodies in prevaccination sera recognizing several antigens in Western blots, although vaccination did not increase such reactivity. Normal serum samples were used as positive controls and indicated that anti-IgG1 and anti-IgG2 antibodies can actually detect IgG1 and IgG2 in Western blots (Fig. 3C).

As low antibody reactivity was found in general against tumor antigens, and to disclose whether this was due to a deficiency of this group of patients to mount an efficient humoral response or to a weak immunogenicity of tumor antigens, the sera reactivities against strong antigens such as those present in BCG were measured. Every patient received BCG as an adjuvant to VACCIMEL, and all had a basal titer against BCG antigens, ranging from 1/55 to 1/325, as measured with anti-IgG + IgA + IgM antisera (Table 3). After vaccination, the placebo group did not change significantly the reactivity (post/pre ratio = 1.7; $P = 0.7$). Instead, and contrasting with the irregular development of antitumor antibodies, all the groups that received rhGM-CSF increased their post/pre ratio of antibody titer to 36.7, 25.8, 59.1, and 10 for groups 2, 3 ($P = 0.03$), 4 ($P = 0.03$), and 5 ($P = 0.06$), respectively (Table 3). Western blots were developed with anti-human IgG1 and IgG2 antibodies, and every tested serum reacted much more intensely with anti-IgG2 than with anti-IgG1 antibody. The most abundant antigens were 38 kd and 65 to 68 kd, with several less intense bands also being detected. As an example, anti-BCG antibodies detected in patients P.B., G.Z., and N.B. are shown (Fig. 3D).

In contrast, anti-rhGM-CSF antibodies were not detected by Western blot in the sera of vaccinated patients (not shown).

Cytokine Serum Concentration

As discussed under Introduction, we decided to investigate in our group of patients the serum levels of IL-10 and IL-12, anti-inflammatory and proinflammatory cytokines, respectively.[18] In every case, serum IL-10 levels were under the detection limit (< 7.5 pg/mL). Instead, IL-12 was readily detectable, and the median concentration in 11 normal donors was 82.9 pg/mL (n = 12). In the patient's sera, the median levels before and after vaccination were 62.4 pg/mL (range 0 to 214 pg/mL) and 75.8 pg/mL (range 31.5 to 500 pg/mL), respectively. The difference was not statistically significant ($P = 0.46$). In addition to this, IL-12 levels increased in patients without evident disease from 85.3 pg/mL before

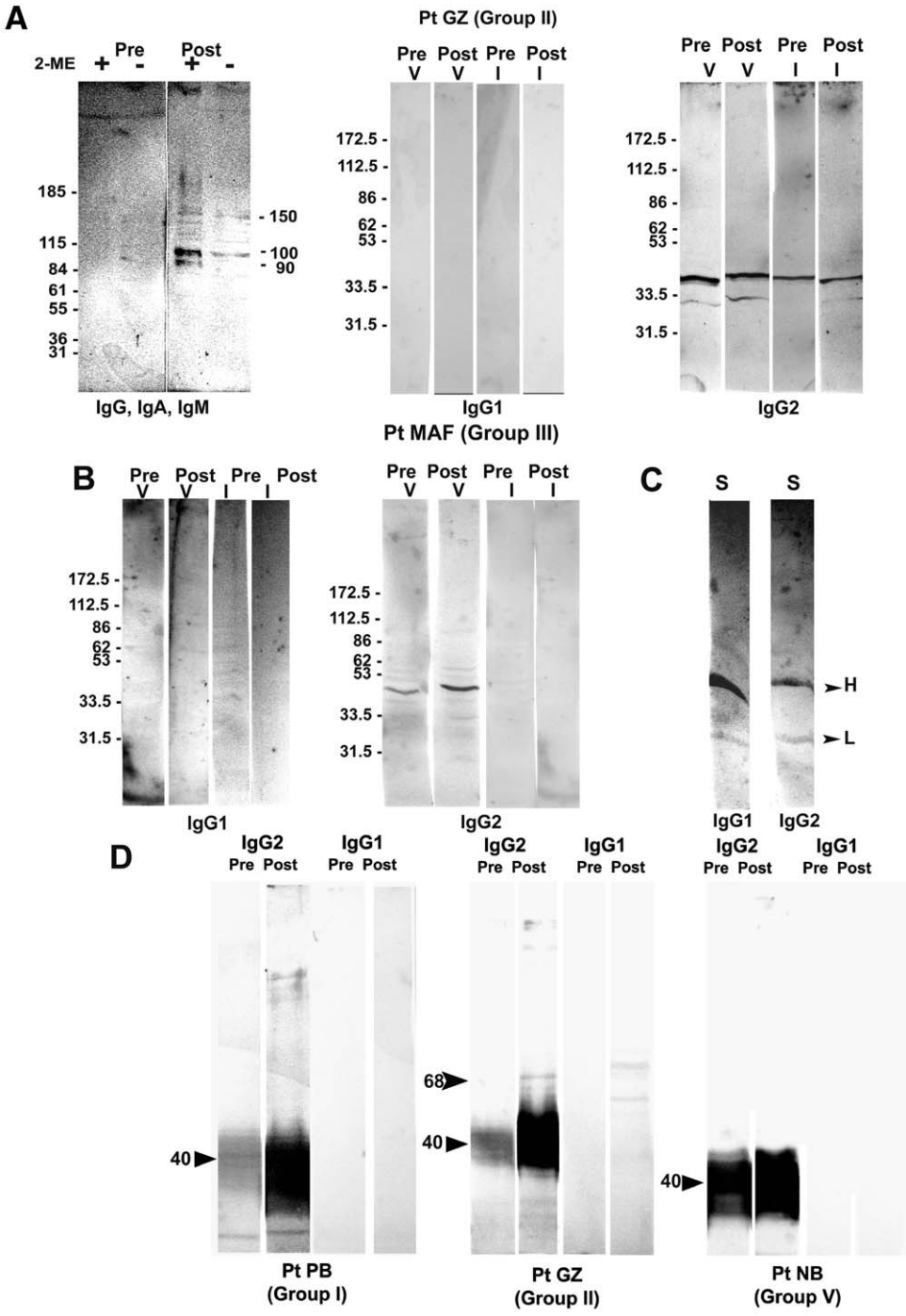

FIGURE 3. Humoral immune response measured by Western blots. VACCIMEL cell extracts were fractionated in SDS-PAGE under reducing (+2-ME) or nonreducing (− 2-ME) conditions and Western Blot was performed with sera diluted 1/10. Pre indicates prevaccination sera; Post, postvaccination sera; V, cell extracts were prepared from VACCIMEL; I, cell extracts were prepared from IIB-BR-G. A, Patient G.Z. Left panel: the secondary antibodies were anti-IgG, IgA, and IgM. Central panel: the secondary antibody was anti-IgG1. Right panel: the secondary antibody was anti-IgG2. B, Patient M.A.F. Left panel: secondary antibody was anti-IgG1. Right panel: the secondary antibody was anti-IgG2. C, Reactivity of secondary antibodies anti-IgG1 and IgG2 were tested with normal sera. D, Anti-BCG antibodies were detected by Western blots in prevaccination and postvaccination sera developed with antihuman IgG1 and IgG2 isotypes as described under Methods.

vaccination to 143.1 pg/mL after vaccination, although this difference was not statistically significant (mean values). The opposite was found in progressing patients, in which the IL-12 levels were slightly higher before vaccination than after vaccination (mean 75.8 vs. 67.2 pg/mL). However, when the patients were grouped by their clinical outcome, the median IL-12 concentration in postvaccination sera was significantly lower in those patients with progressive disease than in those who remained disease free (medians 61.6 vs. 89 pg/mL, $P = 0.01$) (Figs. 4, 5).

Lymphocyte Proliferation in Response to PHA

The patients enrolled in this phase 1 clinical trial were not selected by their HLA class I haplotype, and thus HLA-restricted tumor-specific responses could not be studied. Instead, as a measure of immune competence, we analyzed the ability of a patient's lymphocytes to

TABLE 3. Anti-BCG Titer†

Group	Prevaccination	Postvaccination	Post/Pre Index	*P*
1	325 ± 450	550 ± 520 (n = 4)	1.7	0.7
2	100 ± 0	3666 ± 2309 (n = 3)	36.7	ND*
3	77.5 ± 45	2000 ± 2000 (n = 4)	25.8	0.03
4	55 ± 52	3250 ± 4500 (n = 4)	59.1	0.03
5	77.5 ± 45	775 ± 450 (n = 4)	10.0	0.06

*P value could not be calculated for group 2 as n < 7.

†Titers were calculated after testing by Western blot patient's sera dilutions with BCG extracts as described.

The post/pre index is the ratio of the corresponding titers. P values < 0.05 were considered statistically significant (Wilcoxon rank sum test).

proliferate in response to PHA before and after vaccination. Only 50% of the patients had available lymphocytes to be evaluated. We found 3 different patterns of response: (i) patients in which prevaccination and postvaccination lymphocytes were responsive to PHA (group 1: M.G., M.R.C., A.S.S.; group 2: G.Z.). Patients M.G., M.R.C., and G.Z. are melanoma-free 35+, 34+, and 30+ months after starting treatment. G.Z. developed a low-grade astrocytoma of the optic nerve (confirmed by biopsy) 29 months after starting the vaccine protocol. Patient A.S.S. died as a consequence of brain metastases; (ii) patients in which prevaccination lymphocytes were not responsive to PHA but which recovered its responsiveness after vaccination (group 3: M.A.F., group 4: A.F.A., and group 5: E.D.). M.A.F. and H.D. are disease free 27 and 23 months, respectively, after starting treatment, whereas A.F.A. had progressive disease; (iii) patients in which lymphocytes did not respond to PHA or diminished their response after treatment (group 3: J.V. and group 4: J.S.); these patients progressed and died 4 and 6 months after starting treatment.

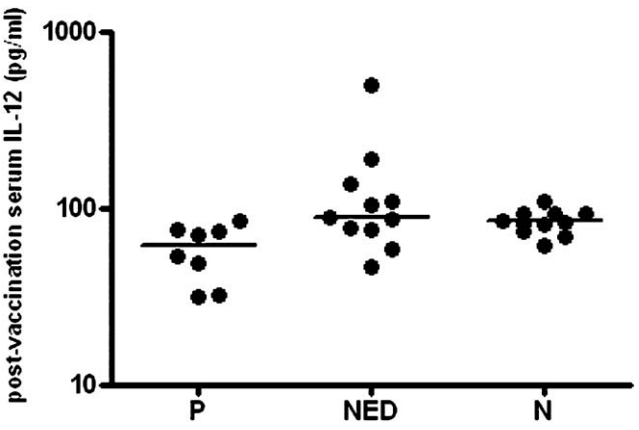

FIGURE 4. Serum IL-12 concentration. Postvaccination sera were obtained 3 weeks after the fourth vaccination and serum IL-12 concentrations were determined by ELISA. The values were compared in patients grouped by their clinical outcome: progression (P) (n = 8) versus NED (n = 11) (minimal follow-up 21 mo), along with a group of untreated volunteer's sera (N) (n = 11). The P versus NED medians had a significant statistical difference ($P = 0.01$, Wilcoxon rank sum test). The horizontal line placed in each group represents the median.

Characterization of Lymphocyte Infiltration in Melanoma Metastases

In 2 patients (A.S.S. and H.I.), cutaneous metastases developed after vaccination and biopsies were obtained. In patient A.S.S. (VACCIMEL + placebo), the extent of tumor necrosis was remarkable even when the tumor was small (3 × 1 mm), which in our experience is not an usual finding. $CD8^+$ lymphocytes briskly infiltrated the tumor, whereas scarce $CD4^+$ lymphocytes were observed. $CD1a^+$ cells were quite abundant in the overlying epidermis (Langerhans cells) and within the tumor, where they could be observed surrounded by lymphocytes. A previously excised lesion from the same patient was a lung metastasis, which only displayed a scarce lymphocytic infiltration. Unfortunately, this patient died a few months after completion of the trial due to brain metastasis.

Patient H.I. (VACCIMEL + 600 μg rhGM-CSF) developed 2 cutaneous metastases after treatment, one in the axilla and the other in the back. We found brisk infiltrates of $CD20^+$ B lymphocytes and $CD8^+$ T lymphocytes, occupying adjacent but nonoverlapping zones of the tumor. No $CD4^+$ T lymphocytes or $CD1a^+$ DCs were detected in this metastatic lesion. After completion of this study, the patient continued to develop cutaneous metastases.

DISCUSSION

The two end points of this work were to determine the toxicity of adding different doses of rhGM-CSF to VACCIMEL and BCG, and the response of the immune parameters to the vaccine. With respect to the toxic effects of the association, they were very mild. The most common toxicity was local inflammation at the vaccine site, which was excessive and led to a reduction in the BCG dose in 5/20 patients; the distribution of these patients among the different groups suggests that BCG toxicity was not related to the GM-CSF dose. The addition of rhGM-CSF to VACCIMEL was well tolerated; grade 2 thoracic pain and abdominal cramps appeared only at the 600 μg dose, and rapidly subsided. No serious adverse effects were detected. With respect to the immune parameters, we tried to detect cellular and humoral immune responses against tumor antigens. DTH is one of the methods usually used to determine cellular immunity, and higher intensities of the reaction are correlated with a favorable outcome in melanoma patients.[15] There was a statistically significant increase in DTH by adding rhGM-CSF to VACCIMEL, attaining a maximum at the 400 μg dose. In this study, we did not routinely obtain biopsies of the vaccination site, the DTH site, or the tumor sites before or after vaccination. Although anecdotal, we describe that 2 patients (A.S.S. and H.I.) developed cutaneous metastasis after vaccination, which were excised and analyzed for lymphocyte infiltration. Although small in diameter (3 mm),

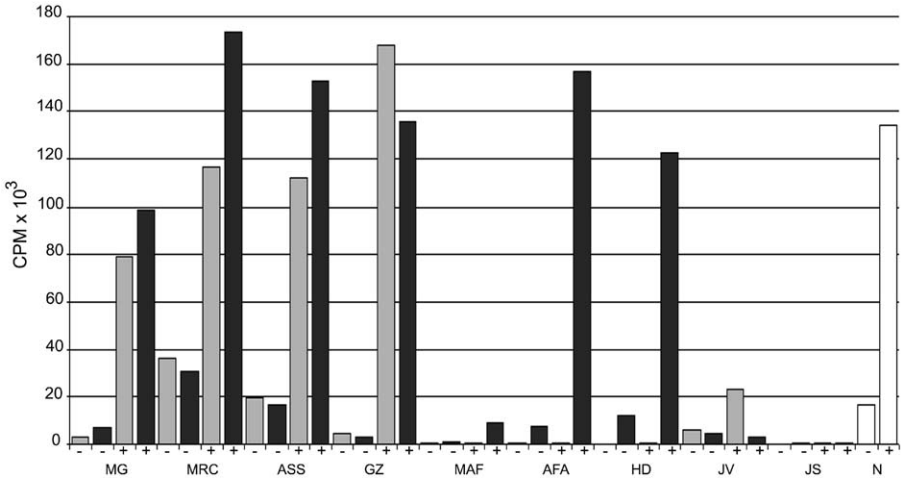

FIGURE 5. Lymphocytes proliferative response to PHA. The ability of the patient's lymphocytes to proliferate without PHA (−) and in response to PHA (+), before and after vaccination, was assayed by (^{3}H)dThd incorporation as described under Methods. The patients whose lymphocytes were analyzed were M.G., M.R.C., A.S.S. (group 1), G.Z. (group 2), J.V., M.A.F. (group 3), A.F.A., J.S. (group 4), and E.D. (group 5). Prevaccination lymphocytes are shown as clear bars and postvaccination lymphocytes as dark bars. Each sample was evaluated in triplicate and bars represent the mean value. As a control, the means from 3 normal donor samples, also evaluated in triplicate, are shown as white bars.

A.S.S.'s metastasis displayed extensive tumor necrosis (> 50%). Characterization of the infiltrating cells by immunohistochemistry revealed an accumulation of $CD8^+$ T cells, and abundant DCs were also observed. A lung metastatic lesion of the same patient before vaccination was minimally infiltrated with lymphocytes. The cutaneous metastases of the other patient (H.I.) were heavily infiltrated with $CD20^+$ B lymphocytes and $CD8^+$ T cells. We suggest that the observed tumor infiltration with lymphocytes is due to vaccination, as melanoma metastases from nontreated patients have been repeatedly shown to be devoid of lymphocytic infiltrates.[8,9]

The activation status of prevaccination and postvaccination peripheral blood T lymphocytes has also been investigated. As HLA-A02 positivity was not an enrollment requisite, and taking into account that most peptides derived from melanoma antigens (gp100, MART-1, MAGE) are presented through this haplotype, we were not able to measure specific lymphocyte responses to these antigens. Instead, it was decided to use PHA as a nonspecific polyclonal stimulator of T lymphocytes. We observed that some patients had normal responses to PHA before and after vaccination. Another group displayed a poor response before vaccination, which recovered after vaccination. Finally, a third group of patients had a low response to PHA before and after vaccination, and the clinical course of these patients was unfavorable. A low lymphocyte response to PHA has been described by several authors to confer the worst prognosis in patients with solid tumors.[29] Also, a low lymphocyte proliferative response to PHA was observed before vaccination in melanoma patients vaccinated with a HLA-A1–restricted MAGE-3 epitope peptide.[30] Perhaps more related to the functional significance of the restoration of lymphocytes' ability of being polyclonally stimulated, it has been shown that in metastatic melanoma patients vaccinated with an autologous melanoma peptide and particulate hepatitis B antigen-exposed immature monocyte-derived DCs, development of melanoma-specific cellular immunity and T-cell responsiveness to PHA were greater in the group of patients responding to the particulate hepatitis B antigen.[31] Therefore, general immune competence may be a determinant of the response to immunologic treatments, and it should be assessed in future immunotherapy trials. Further studies are needed to assess if a diminished PHA stimulation reflects a lymphocyte population imbalance or if it is a general property of diverse lymphocyte subpopulations.

When the antitumor antigens antibody response was measured by FACS analysis, a basal anti-VACCIMEL reactivity was detected in 8/19 patients. This is not surprising, as several of the known tumor antigens were identified through the analysis of untreated patient's sera by the SEREX technique.[32] Basically the same results were obtained when the sera were analyzed by Western blots, as 9/19 patients had antimelanoma antibodies even before vaccination. In some patients, IgG and IgM antibodies against 100 and 90 kd antigens were detected. A 90-kd glycoprotein with antigenic properties has been previously described by Morton and colleagues.[33] The lower detection rate of the 90-kd antigen observed in our group of patients may be due to technical differences, because those authors used a purified 90-kd antigen to perform Western blots. In some patients, 33-kd and 40-kd antigens were also detected, whose nature is still unknown. In some patients, the reactivity against melanoma antigens was rather specific, as their sera did not react against a human breast cancer cell line. In other patients, cross-reactivity was found, but this point was not further investigated. However, and in general, combining our FACS and

Western blot analysis, we did not observe a great increase in serum antibodies to VACCIMEL after vaccination. This was not due to an inherent inability of the patients to mount humoral responses, because we found rising titers of anti-BCG antibodies with increasing doses of rhGM-CSF, the maximum titer being attained at the dose of 400 μg GM-CSF per vaccine. The main recognized BCG antigens were a sharp band around 68 kd that could correspond to the highly antigenic heat shock protein 65 from BCG,[34] and a more diffuse band around 40 to 38 kd, similar to a BCG antigen previously reported.[35] Information regarding anti-BCG antibodies is scant, probably because protective immunity against mycobacteria seems to depend on antigen-specific T cells and not on antibodies.[36] Most of the antibodies were of the IgG2 isotype, which has been shown to be induced by IFN-γ.[37] Therefore, and as previously described,[33] the reactivity to melanoma antigens and to BCG seem to follow different pathways.

With respect to the measurement of cytokines in serum, we did not find the immunosuppressive cytokine IL-10 in any of the patients. IL-10 has been found to be produced by melanoma cells[38] and it was associated with a shorter survival in melanoma patients.[22] Probably, we have not found this cytokine in our patients because they had no evident disease or had minimal tumor mass. In contrast, we have detected serum levels of IL-12, a heterodimeric cytokine composed of unrelated 35 and 40 kd subunits. It is interesting to note that the circulating levels of IL-12 were lower in patients who progressed or died, and who were mostly at stage IV of the disease, than in patients who remained disease free after a maximum follow-up of 35 months, and who were in their majority at stage III ($P < 0.01$). Although these potentially interesting findings must be confirmed in larger number of patients of the same disease stage, they suggest that progressing patients have lower levels of systemic IL-12, which could reflect a reduced proinflammatory profile. It could be therefore speculated that an immune balance shifted toward a T_H1 response may confer a better prognosis to melanoma patients. It is yet not possible to determine which of the components of the vaccine is responsible for the elevated IL-12 levels, but it does not seem to depend exclusively on GM-CSF, because even in group 1, which received placebo, IL-12 levels increased after vaccination (not shown). IL-12 deficiency has been associated with recurrent infections,[39] whereas elevated IL-12 levels have been found in patients prone to autoimmunity, such as women with a history of recurrent miscarriages[40] and patients with systemic erythematous lupus.[41]

To summarize, the increased DTH against tumor antigens, the fact that some patients developed IgG2 but not IgG1 antitumor antibodies, the presence of IL-12 and the absence of IL-10 in serum, and the increase in polyclonal T-lymphocyte response to PHA in some patients, strongly suggest that a predominant T_H1 environment was triggered in some patients by the vaccination process.

There are several advantages in adding soluble rhGM-CSF to the vaccines with respect to cellular transfection with the cytokine gene. In the first place, the use of retroviral or adenoviral vectors engineered to secrete GM-CSF is avoided. Therefore, no special facilities for viral infection are needed and the procedure may be performed in a general hospital. Moreover, the cytokine dose may be calculated with precision, and adequate local concentrations may be achieved. This is especially important, because supra-optimal doses of GM-CSF have been found to have immunosuppressive properties, probably due to a systemic effect leading to the recruitment of myeloid suppressor cells.[42] This could explain why, at the 600 μg dose per vaccine, a lower DTH and diminished antitumor and anti-BCG antibodies were found. The half-life of GM-CSF in mouse skin is around 2 hours (Gazzaniga et al, unpublished results) and therefore, with the optimal found dose of 100 μg/d/vaccine injected in 0.5 mL volume (around 200 μg/mL), 30 ng/mL (around 2 nM) would still remain at the injection site 24 hours after injection, well above the GM-CSF high-affinity K_d binding to the receptor (170 pM)[43] and of the concentration of 20 to 80 pg/mL needed to support 50% growth of an erythroleukemic cell line growth.[44] We conclude that in the allogeneic vaccine setting, soluble GM-CSF administrated by subcutaneous injection can be exploited as an adjuvant to safely and effectively augment tumor immunity. With respect to the evolution of the stage III patients, it is noticeable that, with follow-ups that vary between 21 and 35 months, 7/10 patients (70%) are still disease free. In contrast, 7/8 patients with stage IV melanoma had progressive disease. This suggests that the combination of VACCIMEL, BCG, and GM-CSF would attain its maximal efficacy in high-risk patients with loco-regional disease, such as stages IIB, IIC, and III after surgery, although the clinical efficacy of this combination should be explored in larger, randomized clinical trials.

ACKNOWLEDGMENTS

This work is dedicated to our patients. J.M. is a member of CONICET, E.vE. is a Fellow of the University of Buenos Aires, and M.B. and P.M. are Fellows of the Fundación Sales. We thank Dr Esteban Corley (PC-Gene) for the provision of human recombinant GM-CSF; the Hemotherapy Department of the Instituto Alexander Fleming for providing blood from normal volunteers, and the nurses of the Day Hospital of the Instituto Alexander Fleming for blood extraction from the patients.

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