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ALLOGENEIC CELLS VACCINE INCREASES DISEASE-FREE SURVIVAL IN STAGE III MELANOMA PATIENTS

A NON RANDOMIZED PHASE II STUDY

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Summary The incidence of melanoma is increasing rapidly, and in many cases the primary tumor is excised after metastatic spreading. In 80% of the cases, the first metastatic site is in regional lymph nodes (AJCC Stage III). After excision of these nodes, the patient is clinically disease-free, but the chances of recurrency vary between 40-80%. Thirty patients with stage III melanoma were treated in a non-randomized Phase II adjuvant trial with a vaccine consisting of a mixture of three allogeneic cell lines: IIB-MEL-J, IIB-MEL-LES and IIB-MEL-IAN (5 x 10^6 cells each). The cells were irradiated (5,000 cGy) and BCG was used as nonspecific stimulant. Before each vaccination (72 hr) the patients received cyclophosphamide (300 mg/sqm). The untreated control group was composed of 24 Stage III melanoma patients. Vaccination started within 60 days after surgery, and patients received 4 vaccinations, one every 21 days and then 1 every two months during the 1st year; 1 every three months during the 2nd year, and 1 every 6 months during the 3rd, 4th and 5th years. The treated group was composed by 19 men (63.3%) and 11 women (36.7%); average age: 47.6 ± 14.1 years (range: 16-70 yr). The control group was composed by 18 men (75%) and 6 women (25%); average age 49.8 ± 14.2 yr (range: 26-73 yr). The median disease free survival (DFS) calculated according to Kaplan-Meier was 7.0 months in the control group vs 20.0 months in the treated group ($p < 0.001$). The results of this clinical trial suggest that treatment with allogeneic cell vaccines increases DFS in stage III melanoma patients.

Key words: allogeneic tumor, anti-melanoma, vaccines

The incidence of skin melanoma is increasing in Western countries more rapidly than any other major cancer[1]. A sequence of events from common nevus to dysplastic nevus to melanoma has been described[2], and the course of the disease is afterwards fairly reproducible in its pattern. When the surgery of the primary lesion is not curative, most patients recur at the regional lymph nodes level (Stage III of the American Joint Cancer Committee, AJCC). At this stage surgery is mandatory, and the prognosis of the disease is adversely determined by the extent of lymph node compromise[3]. Once disseminated, the prognosis of melanoma patients is quite dismal, since the best available treatments offer about 50 % total response rates with only 5-10% of complete responses, and at the expense of considerable toxicity[4-6]. The biology of melanoma offers a few paradoxes. Even if it appears to be an immunogenic tumor, the host is generally unable to reject it. One of the reasons for such inability appears to be the down-regulation by melanoma cells of immune reactivity[7]. Besides, melanoma metastases tend to grow as cellular conglomerates with scarce desmoplasia and lymphoid or

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macrophage infiltration. It is under an adjuvant setting that vaccination[8-15] may help the natural course of the disease, since melanoma antigens would be exposed to antigen presenting cells (APC) under a more favorable context for their processing and presentation and would afterwards elicit systemic immunity. In a previous Phase I study, vaccines composed of a mixture of three allogeneic melanoma cell lines were shown to induce less toxicity and stronger delayed-type hypersensitivity (DTH) than cellular lysates[16]. In this paper a non-randomized Phase II study will be described, in which whole allogeneic irradiated melanoma cells with BCG were used as vaccines. It will be described that the vaccine treated group, composed of 30 stage III melanoma patients, had a statistically significant ($p < 0,001$) increase in the disease-free survival as compared to a control group of 24 patients of a similar clinical stage who did not receive any treatment after surgery.

Materials and methods

Pacient population. A total of 54 patients with histologically confirmed AJCC stage III metastatic melanoma were included into this study. After surgery all patients were disease-free as revealed by clinical examination and CAT scans. Patients were not stratified according to the number of metastatic lymph nodes. Patients with capsular node invasion were irradiated at the site of lymphadenectomy but inclusion into this protocol was not delayed. Vaccinations were started no later than two months after surgery. This study was authorized by the Institutional Review Board of the Alexander Fleming Institute, and all patients gave informed consent to the vaccine treatment.

Patients follow-up. At each vaccination the patients received a complete clinical examination. Before surgery a complete CAT scan was obtained. During treatment, chest X-rays and abdominopelvic sonography were obtained every three months, and a complete CAT scan was repeated at least once a year. The patients of the control group were similarly examined.

Melanoma cell lines. The human melanoma cell lines IIB-MEL-J, IIB-MEL-LES and IIB-MEL-IAN were grown as described[17, 18]

Vaccine preparation. Each vaccine dose was composed of 15×10^6 melanoma cells, comprising 5×10^6 cells of each human melanoma cell line IIB-MEL-J, IIB-MEL-LES and IIB-MEL-IAN. Exponentially growing cells were collected, irradiated with 5,000 cGy and frozen in liquid nitrogen in medium containing 20% fetal bovine serum (FBS) (Gibco, USA) - 10% DMSO. The day of injection, the cells were thawed, washed twice with cell medium and resuspended in 0.5 ml of the same medium. The cells were used within 3 hr of preparation.

Vaccine injection. To the cell volume, 0.1 ml. containig 2×10^6 BCG microorganisms (Strain 1173 P2; Instituto Nacional de Microbiología Carlos Malbran) were added. The patients were i.d. injected in three sites in the arm. When axillary dissection had been performed, the contralateral arm was used.

Vaccination scheme. The vaccines were administered as follows: 4 courses, one every 21 days and 1 course every 2 months for the rest of the first year. During the second year one course every three months was given, and during the third, fourth and fifth year the patients received one course every 6 months. Three days before each vaccine, 300 mg/sqm cyclophosphamide were injected i.v. in bolus. This treatment has been shown to temporarily decrease suppressor lymphocytes[19].

Delayed type hypersensitivity (DTH). Simultaneously with each vaccination, the patients were injected in the forearm with 1/10th of the vaccine content without BCG. The reactivity was read at 10 min, 24 hr and 48 hr. The diameters of erythema and induration were measured at these times, and a reaction greater than 0.5 cm (induration) or 1 cm (erythema) was considered to be positive. When the patient was non-reactive after four vaccinations, 10 U of tuberculin (tuberculin-purified protein derivative, 100 U/ml, Statens Serum Institut, Denmark) were also injected in the same forearm at a proximal site to check if lack of reactivity was general or restricted to melanoma antigens. DTH was evaluated as follows: ± : erythema and/or induration 0,5 - 1 cm or irregular reactivity; +: erythema and induration 1-2 cm; ++: erythema and induration > 2 cm; +++: erythema and induration > 2 cm or hemorragic reaction.

Humoral response. It was determined by Western Blot analysis. The pre-immunization serum of patients served as their own controls, and the antigen source was an extract of the cellular vaccines. The extract was prepared by thawing a vaccine, washing as described and centrifuging. The cell pellet was resuspended in lysis buffer: 50 mM Tris-ClH pH 7.5, 150 mM NaCl, 1% NP40, 0.02% Na_3 N, 5 mM EDTA and 1 mM PMSF. After incubating during 20 min at 4ºC, the lysate was centrifuged for 20 min at 20,000 rpm. The supernatant was preserved and its protein content was determined[20]. Ten µg protein per lane of the lysate were electrophoresed in 12% PAGE gels and transferred to nitrocellulose membranes (Sigma). After transfer, the membranes were blocked with 3% dried bovine milk (Molico) in PBS. The sera were diluted 1/50 and pre-incubated with FBS overnight at 4° C to precipitate anti-FBS antibodies. After centrifuging at 10,000 rpm for 30 min, the pre-adsorbed

sera were incubated with the membranes overnight at 4° C. The membranes were developed with a 1/10,000 rabbit anti-IgG and anti-IgM antiserum, alkaline phosphatase bound (Jackson), using the BCIP-NBT system (Gibco).

Statistical analysis. The results were reported according to Kaplan-Meier[21] and statistical significance was obtained by the Wilcoxon rank test[22].

Results

Treated population. Thirty patients were entered into adjuvant treatment with vaccines as described under Methods. The relevant clinical characteristics of this group of patients are shown in Table 1. The average age was 47,6 ± 14,1 years (range 16 70 years), and the group was composed by 19 men (63.3%) and 11 women (36.7%). This group of patients received a total of 286 vaccines, with an average of 9.5 ± 5.0 vaccinations per patient. The most common toxicity was observed at the site of vaccination and consisted in redness and swelling. Ulceration at the vaccine site was frequent and took weeks to months to heal, leaving generally a scar. DTH varied between lack of reaction to a potent, sometimes hemorragic, reaction.

Control population. The mean age of the 24 patients composing the control group was 49.8 ± 14.2 years, and it was composed of 18 men (75%) and 6 women (25%). The control and treated groups were therefore comparable in age and sex distribution. The patients of the control group did not receive any treatment after lymph nodes resection.

Disease-free survival (DFS). The five year DFS of these patients was updated in January 1997, the shortest follow up of disease-free patients being 13 months. The DFS of the control and treated groups were plotted according to Kaplan-Meier[21] (Figure 1). It can be observed that in the control group the median time to progression was 7.0 months whereas in the treated group the median time to progression was 20.0 months. In the control group there are no censored observations, whereas in the treated group there are still 12 censored observations. Twenty months after surgery, in the treated group there are still 10/30 patients without relapse (33.3%) vs only 1/24 patients in the control group (4.1%). The difference in DFS between both groups was highly significant ($p < 0,001$). It should be noted that two patients of the treated group (#5 and #13) recurred exclusively at the CNS.

TABLE 1.– *Vaccine Treated Patients. DFS and Immune Reactivity*

The patients were treated with allogeneic vaccines as indicated under Methods. Age and sex are indicated in the table. DFS is considered from the time of lymph nodes surgery. DTH and anti-melanoma antibodies (Abs) were assayed as described under Methods. ND: not determined; 0: absence of reactivity; ± borderline or irregular reactivity; +: light reactivity; ++: medium reactivity; +++: strong reactivity

Patient	Age/Sex	DFS (months)	DTH	Abs
1	45/M	4	+	ND
2	47/M	7	ND	ND
3	59/M	7	0	ND
4	48/M	8	0	ND
5	37/M	8	0	ND
6	70/F	8	0/+	ND
7	35/F	10	+++	±
8	30/M	12	+	0/+
9	34/F	12	++	±
10	61/M	12	±	ND
11	60/M	13	0	ND
12	57/M	13+	++	±
13	45/M	14	+	0
14	45/F	14	+	±
15	33/M	14+	+++	+++
16	67/M	15+	+	+
17	64/M	16	0/+	0
18	42/F	17+	++	+
19	65/F	20	+	ND
20	64/M	20	0/+	±
21	52/M	24	+	+++
22	48/F	27+	++	+
23	55/M	31+	+	0
24	59/M	32	±	+
25	30/F	49	ND	ND
26	16/F	51+	±/+	+
27	48/M	51+	+	+++
28	17/M	52+	+++	+++
29	44/M	52+	0/+	0
30	50/F	65+	++	++

Immune response. To analyze the induction of anti-melanoma antibodies by vaccination, the pre- and post- immunization sera of 20 patients were

analyzed by Western blots. In 16/20 patients (80%) antibody formation could be detected (Table 1). As an example of the reactivity obtained, the Western blots of patients #28, #8, #22 and #27 is shown in Figure 2. Two triplets of 43-47-48 kDa and 55-57-59 kDa were usually detected. A major band of 59 kDa was detected in patients #27 and #28. Instead, in some patients (i.e. patients #13 and #17) no antibody response to vaccination was observed. The sera obtained before immunization were non reactive (Figure 2). Most of the antibody response was of the IgG type and extracts of normal tissues (WBC) were non reactive (data not shown). The sera of two patients (#15 and #28) showed a strong complement mediated cytotoxicity in vitro against the IIB-MEL-LES cells (data not shown).

As it refers to cellular immune response, DTH to melanoma antigens was positive in 19/28 evaluated patients (67.8%), although the intensities were variable, as shown in Table 1. In 5/5 negative patients PPD reaction was also performed to disclose if lack of reactivity was general, and in all of them DTH to PPD was found to be positive.

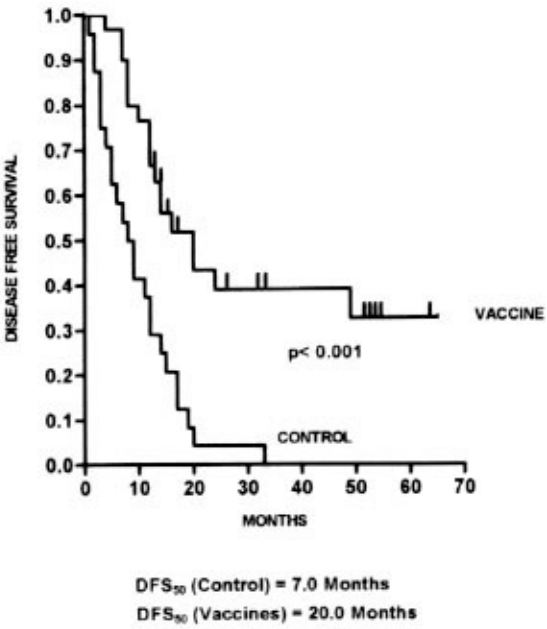

Fig. 1.– Effect of allogeneic vaccination on DFS in Stage III melanoma patients. The DFS of the treated and control groups were plotted according to Kaplan-Meier. The statistical significance was calculated as described under Methods and was found to be highly significant ($p < 0.001$).

Discussion

The results presented in this paper suggest that vaccination with an allogeneic cell vaccine composed of three human melanoma cell lines developed in our laboratory delays recurrence in stage III melanoma patients. It should be emphasized that patients with bad prognosis, such as a large number of metastatic lymph nodes or capsular invasion were also included in our trial. The median DFS in the treated group was 20.0 months, significantly higher than the 7.0 months median DFS in a non treated control group, and compares favorably with the median DFS of 20.7 months in a group treated with interferon-alfa 2b in a much larger multi-institutional trial which also included better prognosis Stage IIB patients[23]. In this paper it has been demonstrated that allogeneic

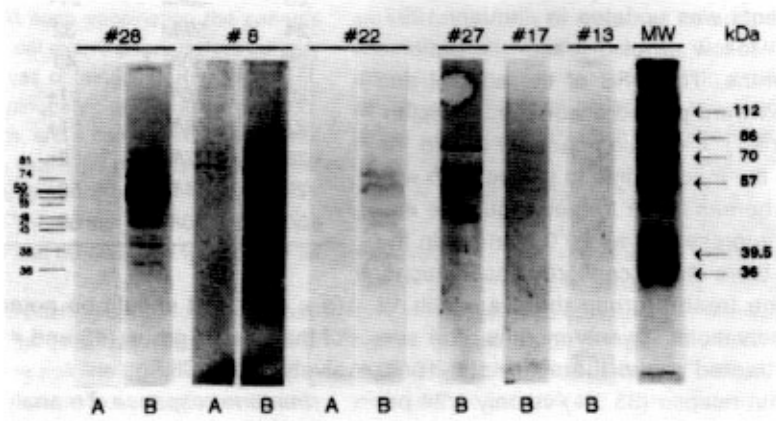

Fig. 2.– Antibodies against melanoma antigens in vaccinated patients. Sera from different patients were obtained before (A) or after several vaccination courses (B). Western blots were performed as described under Methods.

melanoma cell vaccines are able to induce cellular and humoral response against melanoma antigens in a safe and convenient way. Cellular immunity, as measured by DTH, was induced in 19/28 patients (67.8%). We have also demonstrated that 80% of vaccinated patients produce antibodies directed against several melanoma antigens, these antibodies being absent before the start of vaccination. The nature of the respective antigens remains to be ellucidated, as well as the functional significance of the antibodies directed against them. It is worth mentioning that positive skin tests demonstrated a rapid onset (10 min) of reactivity that reached a peak 36 - 48 hr afterwards and then subsided, suggesting a complex reaction that may encompass both an Arthus-type of reaction due to circulating antibodies and a T-cell mediated reaction. It is not yet possible to state whether the prolongation of DFS is due to the induction of cellular or humoral responses because: a) we were not able to demonstrate a clear relationship between DTH intensity and DFS. However, evidence suggests that patients with stronger DTH to melanoma antigens tend to have better evolution than patients with absent or weak DTH (Table 1); b) although anti-melanoma antibodies are clearly induced by vaccines (Fig. 2) it must still be demonstrated that they neutralize the patients' tumor cells. Favoring this assumption, the sera of two patients with strong antibody response (# 15 and # 28) had complement-mediated cytoxicity against IIB-MELLES cells in culture. It should also be noticed that some patients that recurred promptly did not show antibody development (Fig. 2, patients #13 and #17).

It does not seem probable that the prolongation of DFS is due to the adjuvant component of the vaccines (BCG) since BCG alone has not demonstrated any benefit in a great number of adjuvant trials[24]. The benefit observed in DFS can neither be attributed to cyclophosphamide, since this drug, even used at high doses with autologous bone marrow support in Stage III melanoma patients, did not show any benefit in survival[25].

With respect to the mechanism of action of vaccines, studies in murine experimental models suggest that irradiated melanoma cells are digested and processed by APC at the inoculation site. Afterwards, APC would migrate into regional lymph nodes where they would activate naive T and B lymphocytes and thus start systemic immunity[26]. This two-step mechanism would explain why immunization with completely allogeneic tumor cells may confer protective anti-tumor immunity[27].

The formulation of ideal anti-tumor vaccines is still debated. Whereas the use of single peptides or gangliosides as antigens has the advantage of definite composition, they appear to only generate a humoral response[15, 28]. Besides, even if anti-tumor antibodies and/or cytotoxic lymphocytes are developed, their reactivity against single antigens would allow the emergency of resistant tumor cells. Vaccines composed of whole tumor cells are also being actively investigated in other laboratories. They may be composed of autologous unmodified melanoma cells[8], haptenized autologous melanoma cells[9], a mixture of allogeneic cell lines[10], cell lysates of allogeneic melanoma cell lines[12, 13] or proteins purified from allogeneic melanoma cell cultures[14]. In general, all these approaches have demonstrated some benefit in Stage III melanoma patients, although most clinical trials were non-randomized. As shown in this paper, an allogeneic cell vaccine has the ability to generate cellular and humoral immunity directed towards several antigens. Recent evidence in model systems suggests that the multiplicity of foreign antigens, both histocompatibility and tumor associated, does not appear to overwhelm the immune system and diversify the immune response[27].

The continuation of this work will necessarily involve randomized trials in larger groups of patients to confirm the results presented here. Besides, further improvements in the adjuvant management of stage III melanoma patients are needed to avoid or delay the rapid recurrence that takes place in a large number of patients during the first two years after surgery.

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Resumen

Vacuna de células alogeneicas prolonga el período libre de enfermedad en pacientes con melanoma estadio III. Estudio de fase II no randomizado

La incidencia del melanoma está en rápido aumento y en gran número de casos la extirpación del tumor primario se efectúa cuando ya ha metastatizado. El primer sitio de metástasis es en 80% de los casos los ganglios linfáticos regionales (Estadío III). Cuando éstos son extirpados, el paciente queda libre de enfermedad clínica, pero la chance de recurrencia oscila entre el 40-80%. Sobre 30 pacientes en este estadío se efectuó un ensayo clínico de adyuvancia con vacunas alogeneicas de melanoma, cada una compuesta por 15 x 10^6 células de melanoma irradiadas (5000 cGy) (partes iguales de las líneas celulares IIB-MEL-J, IIB-MEL-LES e IIB-MEL-IAN), utilizando BCG como estimulante inespecífico. Antes de cada vacuna (72 hr.) los pacientes recibieron ciclofosfamida (300 mg/m^2). El grupo control estuvo compuesto por 24 pacientes de melanoma estadío III que no recibieron tratamiento. Las vacunaciones comenzaron dentro de los 60 días post-cirugía: 1º año: 4 cursos, uno cada 21 días y luego 1 curso cada 2 meses; 2º año: 1 curso cada 3 meses; 3º, 4º y 5º años: 1 curso cada 6 meses. Composición del grupo tratado: 19 hombres (63,3%) y 11 mujeres (36,7%), edad promedio 47,6 ± 14,1 años (rango 16-70 años). Composición del grupo control: 18 hombres (75%) y 6 mujeres (25%), edad promedio 49,8 ± 14,2 años (rango 26-73 años). La mediana de la sobrevida libre de enfermedad fue de 7,0 meses (grupo control) y 20,0 meses (grupo tratado), diferencia altamente significativa ($p < 0,001$). En las condiciones de este ensayo clínico se sugiere que el tratamiento con vacunas alogeneicas aumenta la sobrevida libre de enfermedad en pacientes con melanoma estadío III.

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It is providencial that the youth or man of inventive mind is not "blessed" with a million dollars. The mind is sharper and keener in seclusion and uninterrupted solitude. Originality thrives in seclusion free of outside influences beating upon us to cripple the creative mind. Be alone that is the secret of invention: be alone, that is when ideas are born.

Es conveniente que un joven con mente inventiva no posea un millón de dólares. Su mente será mas aguda y alerta en reclusión y completa soledad. La originalidad prospera en el aislamiento libre de influencias externas. Estar sólo - éste es el secreto del inventor: estando sólo, ahí es cuando brotan las ideas.

Nikola Tesla (1856 - 1943)