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Effect of BCG on Concanavalin A-induced Suppressor Cell Activity and Lymphocyte Stimulation in Stage I Melanoma

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Suppressor cell activity was determined in 14 patients with stage I melanoma, treated with or without adjuvant Bacillus Calmette-Guerin (BCG) immunotherapy, and in 27 normal healthy volunteers. An *in vitro* test system was used in which peripheral blood mononuclear cells when stimulated with concanavalin A (ConA) significantly suppress proliferative responses of fresh autologous mononuclear cells. In addition, lymphocyte stimulation capacity to optimal and suboptimal concentrations of phytohemagglutinin (PHA) was determined in 44 BCG treated or not BCG treated melanoma patients and in 40 normal individuals.

ConA induced suppressor cell activity was significantly ($p < 0.02$) impaired in BCG treated melanoma patients (21.3 ± 3.1% suppression) when compared to not BCG treated patients (39.8 ± 5.6%) or to normals (38.3 ± 9.3%). Lymphocyte stimulation capacity was depressed in all melanoma patients when suboptimal concentrations of PHA were used but was found to be not significantly altered at optimal concentration of PHA.

The present study reveals that BCG immunotherapy impairs ConA induced suppressor cell activity in melanoma patients but does not influence lymphocyte stimulation capacity.

A variety of suppressor cell systems regulate virtually all immunological processes [1]. In tumor bearing individuals suppressor cells appear to be involved in the immunological enhancement of tumor growth [2–5].

Bacillus Calmette-Guerin (BCG) is one of the most widely used biological adjuvants in the immunotherapy of malignancies and has been shown to modify the immune responses [6]. Consequently, several studies have been performed to investigate the effect of BCG on suppressor cell activity.

In mice, BCG infection activates natural supressor cells [7] and induces the development of macrophage-like suppressor cells [8], capable of inhibiting cytotoxic T cell generation [9].

The present study was performed in order to investigate the influence of BCG on suppressor cell activity in melanoma patients. We have used an *in vitro* test system in which peripheral blood mononuclear cells (MNC) when stimulated with concanavalin A (ConA) suppress proliferative responses of fresh autologous responder MNC [10, 11].

In addition, lymphocyte stimulation capacity was determined in melanoma patients, treated with or without BCG.

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Abbreviations:
BCG: Bacillus Calmette-Guerin
ConA: Concanavalin A
MNC: Mononuclear Cells
NM: Nodular Melanomas
SSM: Superficial Spreading Melanomas
PHA: Phytohemagglutinin

MATERIALS AND METHODS

Patients

A total of 111 individuals was investigated, 44 patients with malignant melanoma, 13 males and 31 females, mean ($\bar{x}$) age 50.48 ± 2.4 yr (± standard error of the mean, SEM) and 67 sex- and age-matched normal healthy volunteers. In all patients, the primary melanoma lesion was excised with a margin of at least 5 cm on either side, followed by selective regional lymphadenectomy. Histologically, the tumors were classified as nodular melanomas (NM; 29 patients) and superficial spreading melanomas (SSM; 15 patients). The invasion of the tumors into the dermis differed from level 3 to level 4 according to Clark et al [12].

No microscopic evidence of metastases was observed in the regional lymphnodes and thus all the patients exhibited stage I of the disease. No further progression of melanoma was observed during the time the study was performed. Four weeks after surgery 22 prospectively randomized patients ($\bar{x}$ age 48.9 ± 3.4 yr; 15 NM; 7 SSM) were started on a BCG immunotherapy (Immuno BCG, Institute Pasteur, Paris, France). BCG was applied intradermally in weekly intervals (dosage 4–6 × 10^8 BCG). The remaining patients (8 males, 14 females; $\bar{x}$ age 52 ± 1.5 yr; 14 NM, 8 SSM) did not receive any adjuvant immuno- and/or chemotherapy at all.

The blood samples for lymphocyte stimulation with phytohemagglutinin (PHA) were taken in the BCG treated patients 9.7 ± 1.5 mo after surgery and in the patients treated by surgical excision alone (without immunotherapy) 8.5 ± 1.2 mo after operation.

Suppressor cell activity of MNC was determined in 7 female patients out of each group. Blood samples were taken in the BCG-treated patients ($\bar{x}$ age 53.1 ± 6.5 yr; 3 NM, 4 SSM) 7.4 ± 1.4 mo after surgery and in the BCG untreated patients ($\bar{x}$ age 48.8 ± 5.1 yr; 4 NM, 3 SSM) 12.4 ± 2.6 mo after operation.

Suppressor Cell Assay

Mononuclear cells: Mononuclear cells (MNC) from heparinized peripheral blood of patients and controls were isolated using Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation method of Boyum [13]. Cells at the interphase were harvested, washed and resuspended in medium RPMI 1640 (Flow Laboratories, Irvine, UK), supplemented with 10% normal human serum of a single pool, used in all experiments.

Activation of MNC with ConA (first culture): Patients and control MNC, 1 × 10^6/ml RPMI 1640, containing 10% pooled human AB-serum, were cultured for 3 to 4 days (a) with 12, 5 μg/ml of ConA (Pharmacia, Uppsala, Sweden) (=ConA-activated MNC) and (b) without the addition of ConA (=control cells).

ConA-activated and control cells were treated subsequently for 30 min with mitomycin C, 50 μg/ml (Kyowa Hakko Kogyo Co, Ltd., Tokio, Japan), blocking further ConA induced DNA synthesis as seen in the proliferation assay. Afterwards the cells were washed 2 times with 100 mM α methylglucoside in RPMI 1640 to remove ConA, followed by 2 washes in medium alone.

Assay for suppressor cell activity (second culture): ConA-stimulated cells and control cells (first culture) were cultured with fresh autologous MNC (responder cells), respectively. These autologous cells were taken 3 to 4 days after the first culture from the identical individual. In this second culture, 1 × 10^5 ConA prestimulated or control cells were cultured again for 3 to 4 days with and without ConA standard dose I μg/ml in 0.2 ml in flat-bottomed microtest plates.

Thymidine incorporation was measured after terminal 8 hr pulse with 5 μCi/ml of 6-H^3 thymidine (2 Ci/mM; Amersham, UK). Cells were processed on a Skarton cell harvester and incorporation of ^{3}H TdR was measured by a liquid scintillation counter as described previously [14].

Data are expressed as mean desintigrations per minute (dpm) of the mean ± standard error of the mean (SEM). Values of Δdpm were calculated by substracting dpm of unstimulated cultures from dpm of stimulated sultures.

To standardize results and permit evaluation of the degree of suppression the following formula was used:

$$\text{percent (\%) suppression} = \frac{1 - \Delta \text{ dpm ConA}}{\Delta \text{ dpm control}} \times 100$$

Δ dpm ConA represents Δ dpm after the addition of ConA activated MNC to the second culture and Δ dpm control stands for Δ dpm after the addition of control cells (incubated but not ConA activated MNC) to the second culture.

Lymphocyte Stimulation

Lymphocyte stimulation was measured by a whole blood method [15], a test system, exhibiting the advantage of minimal blood requirement and minimal alteration of cells by manipulation and isolation procedures [15]. Purified phytohemagglutinin (PHA) HA 16/17 (Wellcome Research Laboratories, Beckenham, UK) was used as mitogen in optimal (0, 1 mitogen units/ml) and suboptimal (0.005 mitogen units/ml) concentrations.

Statistics

Statistical analysis was performed using the Student's *t*-test. The correlation coefficient (r) was determined by linear regression analysis using least squares fit.

RESULTS

Suppressor Cells

The suppressor cell activity of ConA stimulated MNC ($\bar{x}$ = 21.3 ± SEM 3.1% suppression) of patients with stage I melanoma, treated by BCG immunotherapy was found to be significantly ($p < 0.02$) lower than the suppressor cell activity of ConA stimulated MNC (39.8 ± 5.6%) of patients with melanoma treated by surgery alone (Table I); suppressor cell activity was also found to be significantly ($p < 0.05$) decreased when compared to the suppressor cell activity of ConA stimulated MNC (38.3 ± 9.3%) of 27 normal healthy volunteers (Fig 1). No significant difference was observed between the mean percentage suppression of untreated patients with normals. In addition, no correlation was observed between the percentage suppression and (a) the age of the patients (BCG: correlation coefficient r = 0.11; non BCG: r = 0.03) (b) the time after operation (BCG: r = 0.52; non-BCG: r = 0, 12) and (c) the histopathological type of the tumors, in both BCG and untreated melanoma patients.

TABLE I. *ConA-induced suppressor cell activity in BCG treated (1-7) and in BCG untreated (8-14) melanoma patients*

Pat. #	ConA activation of MNC in first culture period	^{3}H TdR incorporation in response to ConA	
		Δ dpm	% suppression
1	−	3527 ± 157	
	+	2722 ± 105	23
2	−	7404 ± 454	
	+	5902 ± 468	20
3	−	10224 ± 249	
	+	7048 ± 578	31
4	−	6028 ± 249	
	+	5348 ± 427	11
5	−	10936 ± 868	
	+	7406 ± 871	32
6	−	8323 ± 152	
	+	7294 ± 333	12
7	−	3408 ± 232	
	+	2738 ± 381	20
8	−	44701 ± 817	
	+	31009 ± 641	31
9	−	7303 ± 385	
	+	5374 ± 428	26
10	−	41240 ± 2317	
	+	18513 ± 862	55
11	−	7267 ± 547	
	+	5550 ± 492	24
12	−	10304 ± 541	
	+	4536 ± 605	56
13	−	6808 ± 313	
	+	4632 ± 437	32
14	−	16307 ± 981	
	+	7287 ± 482	55

Lymphocyte Stimulation

The log-transformed values of lymphocyte are shown in Fig 2. Using suboptimal concentrations of PHA (0.005 mitogen units/ml) a significantly lower response was observed in BCG-treated melanoma patients (geometric mean ($g\bar{x}$) = 13 × 10^3 dpm/culture; $p < 0.005$) or melanoma patients, treated by surgery alone ($g\bar{x}$ = 20 × 10^3 dpm/culture; $p < 0.05$) when compared to normals ($g\bar{x}$ = 40 × 10^3 dpm/culture). No signifi-

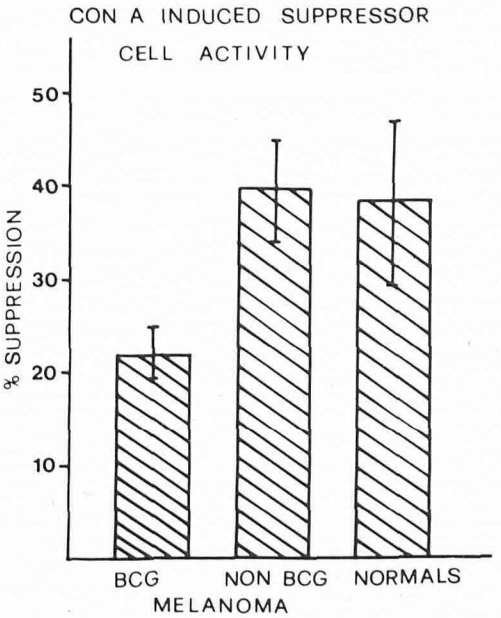

FIG 1. ConA-induced suppressor cell activity is significantly impaired in melanoma patients, treated with BCG immunotherapy when compared to BCG untreated melanoma patients or normal individuals.

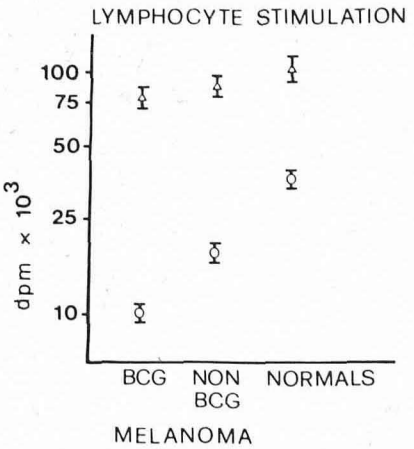

FIG 2. Data represent the log. transformed mean of desintigrations per minute (dpm) ± SEM. Lymphocyte stimulation is depressed in melanoma patients when suboptimal (0.005 mitogen units/ml) concentrations of PHA are used (o), but is found to be normal at optimal (0.1 mitogen unit/ml) concentrations of PHA (Δ).

TABLE II. *Absolute and relative numbers of leukocytes, lymphocytes and monocytes in BCG treated and in BCG untreated melanoma patients*[a]

Patients	n[b]	Leukocytes	Lymphocytes	Monocytes
BCG	22	6346 ± 322	2565 ± 196 (40 ± 1.8%)	165 ± 2.7 (3.4 ± 0.5%)
No-BCG	22	6290 ± 389	2215 ± 168 (35 ± 1.6%)	146 ± 2.5 (2.4 ± 0.4%)
Normal values		4000–10000/mm^3	1500–3500/mm^3 (20–50%)	100–800/mm^3 (2–10%)

[a] Data represent the mean ± standard error of the mean (SEM).
[b] n = number of patients.

cant difference was observed between BCG treated and untreated patients at suboptimal concentrations of PHA.

No significant difference was found in lymphocyte stimulation capacity in the 3 groups of individuals ($g\bar{x}$: BCG melanoma: 80×10^3 dpm/culture, non BCG melanoma: 80×10^3 dpm/culture, normals; 100×10^3/culture) when optimal concentrations of PHA (0.1 mitogen units) were used. Absolute and relative numbers of leukocytes, lymphocytes and monocytes of BCG and not BCG treated patients were found to be within the normal range (Table II).

DISCUSSION

It seems to be generally accepted that tumor patients exhibit several defects of *in vivo* or *in vitro* immune reactions. Frequently these defects are not as pronounced as in classical immunodeficiency states. Impaired lymphocyte stimulation capacity, e.g., is usually only detectable when suboptimal mitogen doses are used [16]. This was also found in the present study. Lymphocyte stimulation capacity was not significantly different between normals, BCG-treated and BCG untreated melanoma patients, respectively, when optimal concentrations of PHA were used. Only at suboptimal concentrations of PHA, lymphocyte stimulation capacity was significantly depressed in BCG-treated as well as BCG untreated melanoma patients, when compared to normals. However, no significant difference was observed between both groups of patients, investigated. These results indicate that BCG immunotherapy does not significantly influence overall lymphocyte stimulation capacity.

However, more recently test systems have been developed which permit a more detailed study of *in vitro* immuno-responsiveness. In tumor immunology increasing interest has focused on suppressor cell assays since there is suggestive evidence, that suppressor cells may undermine an effective antitumor immune response and may lead to immunological enhancement of tumor growth [2–4]. Suppressor cells have been shown to interfere with (tumor) antigen specific killer T cells [17]. Furthermore, studies in mice revealed that suppressor T cells may even influence the outcome of some forms of cancer [3, 18] or may promote the growth of ultraviolet light induced tumors [19]. In human cancer patients increased suppressor cell activity was found in both, cells of the lymphocyte and monocyte population [3, 4]. Increased suppressor T cell activity has been described in progressive osteogenic sarcomas [17]; suppressive monocytes were found in patients with multiple myeloma [20], Hodgkin's disease [21] and lung cancer [22]. Impaired suppressor cell activity has mainly been observed in several inflammatory diseases, including lupus erythemathosus [23], juvenile rheumatoid arthritis [24], inflammatory bowel disease [25] and inflammatory uveal diseases [26].

In most of these studies the ConA induced suppressor cell assay system [10] has been employed. This system measures the suppressive effect of ConA preactivated cells for proliferative response of autologous or homologous responder cells. It thus measures *in vitro*-induced and not spontaneous suppressor cell activity. The suppressor cells detectable in this system have been shown to be of lymphocyte origin [11, 23].

Using this system we found that ConA-induced suppressor cell activity was significantly impaired in BCG-treated melanoma patients when compared to BCG untreated patients and normals, respectively. No significant difference was observed in the suppressor cell activity between normals and BCG untreated melanoma patients. These results indicate that systemically applied BCG impairs ConA inducable suppressor cell activity.

Other authors have used assays which measure suppressor T cell function by the suppression of pokeweed mitogen induced immunoglobulin production as determined by radioimmunoassay [27], or the suppression of the development of plasma cells [28]. Our observations of decreased suppressor activity for ConA induced proliferation do not necessarily implicate a correlation with decreased suppressor cell activity for other T cell functions or for B cell differentiation into plasma cells.

The impaired ConA-induced suppressor cell activity in BCG-treated melanoma patients might be explained by BCG-induced inflammation. As mentioned above, impaired suppressor cell activity has mainly been observed in several inflammatory diseases [23–26]. However, it was only observed to be impaired in the acute phase of the respective diseases, when the inflammatory processes were going on and was normal when the disease was clinically in remission. Thus, it has been postulated that inflammation per se results in impaired ConA-induced suppressor cell activity [25]. Possibly, BCG-induced inflammation also impairs ConA inducable suppressor cell activity.

Another explanation could be that BCG induces the development of antilymphocyte antibodies, capable of inhibiting suppressor cell activity. Antibodies cytotoxic for lymphocytes *in vitro* have been described in more than 30 disorders, including collagene diseases, cancer, renal transplant recipients [29] and even in healthy persons after vaccination with bacterial and viral antigens [30]. Some anti-T cell antibodies have been shown to inhibit suppressor cell activity in patients with systemic lupus erythematosus [23]. In mice, antisera against T cells were shown to abolish suppressor cell activity *in vitro* and to reduce growth of transplantable syngenic tumors *in vivo* [3]. Thus it might be that BCG vaccination also leads to the formation of suppressor T cell (precursor) antibodies and that the observed impaired ConA-induced suppressor cell activity in BCG-treated patients is due to the presence of BCG-induced anti-T cell antibodies.

Furthermore, one could speculate that BCG activates suppressor cells already *in vivo*, and that these *in vivo* activated suppressor cells cannot be further stimulated by ConA *in vitro*. With regard to this speculation it is interesting to note that in mice systemically applied BCG has been shown to activate natural [7] and macrophage-like [8, 9] suppressor cells *in vivo*.

Since increased suppressor cell activity is proposed to enhance tumor growth [2–5] impaired suppressor cell activity should lead to an increased antitumor response of the host. Employing the ConA system [10] we found an impaired suppressor cell activity in BCG-treated melanoma patients. It remains to be proven whether this *in vitro* phenomenon is indeed of relevance *in vivo*, since clinical studies have not uniformly demonstrated a substantial beneficial effect of BCG stimulation in melanoma patient survival [6].

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