

Mechanism of Genetically-Regulated Response to Bacillus- Calmette Guérin Immunotherapy for Bladder Cancer

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ABSTRACT

In this study, we investigated host response to Bacillus-Calmette Guérin (BCG) immunotherapy using the murine MM45T bladder tumor cell line. BALB/c (*Bcgs* allele) and BALB/c.CD2 (CD2) (*Bcgr* allele) mice were seeded subcutaneously with MM45T cells. Treatment began on day 7 with intra-tumor injections of BCG. Significant decreases in tumor growth in BALB/c ($p < 0.05$) but not in the CD2 ($p = \text{NS}$) mice were observed 6, 10 and 14 days following treatment. Investigation of the mechanism(s) underlining the difference in the response between the two strains of mice revealed key points, namely: (i) Intraperitoneal injection of BCG led to a significant increase ($p < 0.05$) in the number of cells recovered 14 days later by peritoneal lavage (in million cells: 5.1 ± 0.7 vs 9.5 ± 0.3 in BALB/c and 5.3 ± 0.4 vs 7.0 ± 0.4 in CD2, PBS vs BCG). (ii) Compositional analysis of the lavages following BCG injection indicated a significant increase in the lymphocyte/macrophage ratio in BALB/c mice as compared to CD2 mice ($p < 0.05$). (iii) Activated peritoneal macrophage production of nitric oxide (NO) was significantly lower ($p < 0.05$) in BALB/c than in CD2 (26.8 ± 1.6 vs 38.3 ± 0.7 μM of nitrite respectively) when challenged with lipopolysaccharide. (iv) No difference in the in vitro cytotoxicity against MM45T cells was observed between BALB/c and CD2 macrophages. Our results suggest that differences in the composition of monocytes infiltrating the site of BCG treatment and in the release of substances such as NO by macrophages may be key determinants of the response to BCG immunotherapy for bladder cancer.

INTRODUCTION

Intravesicular instillation of Bacillus Calmette-Guérin (BCG) is currently the treatment of choice for superficial bladder cancer (1,2). Unfortunately, about 50% of patients with papillary type of carcinoma do not respond to this treatment (3,4). The difference in response is believed to be genetically determined.

Although the efficacy of action of BCG treatment in responsive patients has been well established, the immunologic mechanism(s) of action is (are) still uncertain (5,6). The recruitment and activation of host macrophages is thought to play a pivotal role in killing the bladder tumor cells.

Peritoneal macrophages may be arbitrarily classified as resident, inflammatory, or activated. It is well established that activated macrophages can lyse certain tumor cell lines but not others when cocultured in vitro (7,8). Upon presentation with appropriate stimuli, for example bacterial endotoxin, activated macrophages release substances such as the reactive nitric oxide (NO) radical and tumor necrosis factor- α (TNF- α) (9). The bacterial endotoxin stimulates the expression of the enzyme-inducible nitric oxide synthase (iNOS) accounting for the NO release. Production of NO may be beneficial. Both NO and TNF- α are considered to be important mediators of macrophage tumor cytotoxicity (10,11). However, NO is also a potent vasodilator. As a consequence, during severe bacteremia, overexpression of iNOS can precipitate septic shock (10). It has been shown in human monocytes that LPS-induced release of cytokines such as TNF- α and interleukin-1 (IL-1) can be augmented by prior exposure of the macrophages in vivo to BCG (6).

BALB/c and the congenic BALB/c.CD2 (CD2) mice are genetically identical except at the Bcg gene. BALB/c mice possess the Bcgs allele whereas CD2 mice possess the Bcgr allele. Owing to this genetic difference, the BALB/c mice respond to intravesical BCG treatment of superficial bladder cancer whereas the CD2 mice do not. These two strains of mice therefore provide an excellent model for studying the immunological basis of the genetically observed difference in responsiveness to BCG therapy.

Unfortunately, establishing the presence of and monitoring the sizes of tumors in the murine bladder is both time consuming and requires expensive Magnetic Resonance Imaging (MRI) procedures. However, the ability of the murine bladder tumor cell line, CRL 6420.MM45T, to implant and subsequently grow well at a subcutaneous site is well established in our laboratory for both BALB/c and CD2 mice (unpublished observations). The subcutaneous model of the bladder tumor was thus chosen for the present investigation.

In this study, the responsiveness to BCG treatment was compared for BALB/c and CD2 mice bearing subcutaneous MM45T tumors. The role of macrophages in the genetically-determined response to BCG therapy was also assessed. Macrophages from both strains of mice were activated in vivo by intraperitoneal (i.p.) injection of BCG. Subsequently, the in vitro tumor cytotoxicity, TNF- α and NO production by the BCG-activated macrophages was determined and compared for the two strains of mice.

METHODS

Mice

BALB/c and BALB/c.CD2 (CD2) mice were used in all experiments. These mice were 6-8 weeks old, and either male or female. They were obtained from breeding facilities at the Montreal General Hospital Research Institute. All animal handling was carried out in laminar flow hoods. The mice were fed autoclaved food and water.

Bacillus Calmette Guérin (BCG)

BCG ($6.2 \times 10^6 \pm 1.6$ colony forming units [CFU]) was obtained as lyophilized *Mycobacterium bovis* substrain Montreal (Lot # 9869, IAF Biovac Inc., Laval, QC, Canada). BCG was freshly reconstituted immediately prior to use. For intratumoral treatments, 3×10^7 CFU of BCG in 0.1 ml of sterile water were used. For intraperitoneal treatments, 1×10^6 CFU of BCG in phosphate buffered saline (PBS) was used.

Tumor cells and implantation of subcutaneous tumors

Tumor cells used were MM45T, a urinary bladder carcinoma which arose spontaneously in BALB/c mice (American Type Culture Collection, Rockville, MD, USA). The cells were maintained in a continuous in vitro culture with Dulbecco's modified Eagle medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (HyClone Laboratories, Inc., Logan, UT, USA) and 0.12% gentamycin (Schering Canada Inc., Montreal, QC, Canada). MM45T cells were harvested by washing twice with Ca²⁺- and Mg²⁺- free Hanks' balanced salt solution (HBSS) (Gibco), followed by trypsinization for two to three minutes with 0.5 ml of 0.05% trypsin/EDTA (Gibco). Cells were then resuspended in the growth medium. Cell viability was determined by trypan blue dye exclusion method and was always greater than 90%. Tumors were implanted by subcutaneous injection of 10⁶ MM45T cells in 0.1 ml of DMEM. Tumor diameters were measured in millimeters using vernier calipers.

Tumor Treatments

Subcutaneous tumor treatments were begun on day 7 following tumor implants. Treatment involved intratumoral injection of 3x10⁷ CFU of BCG reconstituted in 0.1 ml of distilled water. Treatments were done every two days for three weeks. Control mice were treated with 0.1 ml of sterile PBS.

Preparation of macrophage monolayers

Peritoneal macrophages were obtained by peritoneal lavage of mice injected 14 days previously with BCG (10⁶ CFU in 0.5 ml PBS). Cells were kept on ice while samples were taken for determining total and differential cell counts. Total cell counts were carried out with a hemacytometer chamber. Differential cell counts were carried out on Cytocentrifuge (Shandon Corporation, Sewickley, PA, USA) preparations of samples stained with hematoxylin and eosin. For the NO and TNF- α assays, cell suspensions were diluted to a concentration of 2x10⁶ macrophages/ml. Aliquots of 100 μ l samples of the diluted cell suspensions were added to a 96-well flat-bottomed plate (Sarstedt, Ville St. Laurent, QC, Canada) and allowed to adhere for 2 hr at 37°C in an atmosphere of 5% CO₂ in air. At the end of the incubation period non-adherent cells were removed by washing three times with 150 μ l warm HBSS (Gibco). This procedure leaves behind a monolayer of adherent macrophages.

NO production assay

Production of NO was estimated by measuring nitrite accumulation in culture medium using the Griess method as described by Green et al. (12). To the macrophage monolayers obtained by the procedure described above, proceeding quickly after the last suction removal of HBSS to prevent cells from drying out, 100 μ l of E. coli O127:B8 LPS (Difco, Detroit, MI, USA) were added at a concentration of 1 μ g/ml in DMEM. After a 24 hr incubation, 50 μ l of supernatant from each well was added to an equal volume of Griess reagent (1:1, v/v, of 0.1% N-naphthylethylenediamine dihydrochloride [NED] in distilled water and 1% sulfanilamide in 5% phosphoric acid) in a 96-well flat-bottomed plate (Sarstedt). Absorbance was measured at 550 nm using a microplate reader. An average of measurements from quadruplicate wells was used in the final analysis. Nitrite concentration was calculated from a standard curve prepared for each experiment using NaNO₂ as the standard.

Measurement of TNF- α production

A double-sandwich enzyme-linked immunoabsorbent assay (ELISA), as described by Sheehan et al. (13), was used to determine the quantity of TNF- α in supernatants of macrophages after 24 hr culture with LPS as

described for the NO assay. Hamster monoclonal antibody to murine TNF- α was purchased from Genzyme (Boston, MA) and rabbit polyclonal antimurine TNF- α was prepared and purified by standard procedures (14).

Macrophage tumoricidal assay

In vitro tumoricidal assays were performed using an adaptation of the colorimetric assay described by Mosmann (15). This procedure uses 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT), a chromogen converted only by live cells. Macrophage monolayers were prepared as described above with the following changes. Peritoneal cells were adjusted to three concentrations of 2.5×10^5 , 5×10^5 , and 1×10^6 macrophages/ml. Aliquots of 100 μ l from each of these adjusted cell suspensions were plated and washed as described above. After gentle suction removal of HBSS from the last wash, proceeding quickly to prevent cells from drying out, 100 μ l of MM45T tumor cells were added to each well at a concentration of 5×10^4 cells/ml. After 48 hrs of culturing macrophages with tumor cells at various effector to target ratios, 10 μ l of stock MTT was added to each well and plates were incubated at 37°C, 5% CO₂ for 4 hrs. Aliquots of 50 μ l of 10% Triton-X in 0.5 N HCl was added to each well and mixed thoroughly to dissolve the dark blue crystals. After approximately 30 minutes of mixing at room temperature to ensure that all crystals were dissolved, the plates were read on a Microplate reader. A test wavelength of 570 nm and a reference wavelength of 630 nm were used. Wells containing macrophage cells or tumor cells alone were used as controls. Percentage tumor cytotoxicity was calculated using the formula:

$$\left\{ \frac{[\text{Absorbance of tumor cells (no macrophage present)} - \text{Absorbance of tumor cells (macrophages present)}]}{[\text{Absorbance of tumor cells (no macrophage present)}]} \right\} \times 100$$

The absorbance of tumor cells in wells containing tumor cells with macrophages was estimated by subtracting the absorbance of macrophage controls.

Statistics

Statistically significant differences between controls and experimental groups were determined using Student's t test. Significance was set at $p < 0.05$. Values are expressed as means \pm standard error of the mean (SEM).

RESULTS

Subcutaneous MM45T tumors

MM45T tumor cells were implanted and subsequently grew equally well in both BALB/c and the CD2 strains of mice. In both strains of mice, there was no significant difference ($p > 0.05$) between the tumor sizes on day 7 post implantation, the day that treatments were begun (Figs. 1A and 1B). BCG treatment did not cause tumor regression but did significantly ($p < 0.05$) inhibit further tumor growth in the BcgsBALB/c mice as compared to controls at all three time points assayed (Fig. 1A). This was not the case in the BcgrCD2 strain of mice. As shown in Fig. 1B, at all time points following tumor implants, tumor sizes in the BCG-treated group were not significantly ($p = \text{NS}$) different from that of the controls.

The data presented in Fig. 1 are tumor sizes calculated using the product of the longest tumor diameter and the (shortest tumor diameter)² as described by Brunda et al. (16). In one BALB/c mouse, not included in the experiment because tumor size was rather small compared to controls at day 7, BCG treatments did in fact lead to complete disappearance of the tumor by the end of the first week of treatment.

Composition of peritoneal cells

In Table 1, the composition of peritoneal cells in mice injected i.p. with BCG is compared with that of control PBS-injected mice. In both BALB/c and CD2 mice, i.p. BCG injection led to a significant ($p < 0.05$) increase in the accumulation of cells in the peritoneum when compared to control mice injected with PBS (experiments 1 and 2). This is reflected in the total numbers of cells.

Not only did BCG stimulate a greater accumulation of peritoneal cells, it also significantly altered the composition of peritoneal cells in BCG-treated as compared to PBS- treated control mice ($p < 0.05$) (experiments 1 and 2). The percentage of total peritoneal cells made up of macrophages was much greater in the PBS-treated control mice than in the BCG-treated mice. As presented in Table 1, the lymphocyte/macrophage ratios of peritoneal cells following i.p. BCG treatment was significantly greater in BALB/c mice when compared to their CD2 counterparts ($p < 0.05$), but the total number of peritoneal cells was not different ($p = NS$) between the two groups (experiment 3).

TNF- α and NO production

Table 2 summarizes the data for the in vitro TNF- α and NO production. Peritoneal macrophages previously activated in vivo by i.p. BCG treatment were primed to produce TNF- α and NO in vitro when stimulated with 1 $\mu\text{g/ml}$ of LPS. Whereas macrophages from both strains of mice had the capacity to produce NO and TNF- α , BALB/c macrophages produced significantly lesser ($p < 0.05$) amounts of NO than the CD2 macrophages. However, the amount of TNF- α produced was not significantly different ($p = NS$) between the two groups.

In vitro cytotoxicity against MM45T cells

At the effector to target ratios of 20/1, 10/1, and 5/1, Fig.2 shows that the in vitro tumoricidal effect of macrophages was not different ($p = NS$) between BALB/c and CD2 macrophages.

DISCUSSION

The efficacy of BCG immunotherapy for superficial bladder cancer is well documented (6). However, the mechanism of BCG's anti-cancer effect remains unclear (1,5). Moreover, a significant proportion of patients do not respond to this mode of therapy. This poses a serious challenge in the decision to administer BCG immunotherapy. At present, there is no way of determining which patients will be responsive.

Intratumoral BCG treatment of subcutaneous MM45T tumors significantly inhibited further tumor growth in BcgsBALB/c mice but not in BcgrCD2 mice as compared to PBS-treated controls (Fig.1). These results support a genetic basis for determining responsiveness to BCG cancer immunotherapy.

It has been proposed that the L-arginine-dependent NO pathway interacts synergistically with the TNF- α pathway in murine macrophage-mediated tumor cytotoxicity in vitro (9). We have compared the in vitro tumoricidal activity, TNF- α and NO production by peritoneal macrophages from both strains of mice in an attempt to provide insights into the immunologic basis of the observed genetic difference in response to BCG therapy. These macrophages had been activated previously in vivo by i.p. injection with BCG. We observed no significant difference in in vitro tumouricidal activity or TNF- α production by activated macrophages between the two strains (Fig. 2). These in vitro results suggest that the genetic difference between the two strains does not lie in the capacity of the macrophages to produce TNF- α . However, TNF- α production in vivo by activated macrophages may still be different between the two strains as a result of the presence of immunomodulatory cytokines that were absent in our in vitro model.

The production of NO by activated macrophages in vitro in response to LPS challenge was significantly different ($p < 0.05$) between the two strains of mice, with the BALB/c mice producing less NO (Table 2). This result suggests that, unlike TNF- α , the genetic difference in response to BCG immunotherapy may be expressed in the capacity of macrophages to release NO following activation by BCG. Further evaluation of such a hypothesis would be provided by measuring in vivo production of NO in both strains during BCG immunotherapy. To our knowledge, this has not yet been done. Although our results suggest that differences in NO production may be an important determinant of response to BCG therapy, the observations made here do not rule out a role for other immunomodulatory molecules or cytokines.

We have found that the composition of peritoneal cells varies significantly between the two strains of mice following an i.p. challenge with BCG (Table 1). The lymphocyte to macrophage ratio was significantly higher in the BALB/c as compared to the CD2 peritoneal cells. We did not, however, determine the phenotype of the lymphocytes. It is important to notice that this in vivo situation is in sharp contrast to the in vitro one used in our cytotoxicity assays. In the latter case, macrophages from both strains were artificially adjusted to desired and equivalent concentrations for ease of comparison and were studied in the absence of lymphocytes. Therefore, we can not assess how the in vivo production of mediators such as TNF- α and NO might be modulated by the presence of lymphocyte-derived cytokines.

The subcutaneous model of bladder cancer used in the present investigation offers many opportunities for further studies on, for example, the role of tumor size on treatment outcome. In addition to genetic factors, the size of tumors at the beginning of BCG therapy may also be an important determinant of response to treatment. This suggestion came from the observation of an isolated case where complete tumor disappearance was observed in one BALB/c mouse following a week of BCG therapy. This mouse was not included in the present study because the size of its tumor at the beginning of therapy had been significantly smaller than those of the rest of the study group. This rather small tumor size was attributed to the mouse not obtaining the full dose of tumor cells during the initial s.c. tumor implantation, possibly due to leakage after injection.

In conclusion, our results suggest that the genetic susceptibility, the composition of effector cells, and the production of relative amounts of NO by activated macrophages are likely to be key players in the mechanism of BCG's anti-cancer effect. However, the exact nature of the interactions among these factors remains to be determined.

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