Antibody Responses to *Bacillus Calmette-Guérin* during Immunotherapy in Bladder Cancer Patients

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**ABSTRACT**

Levels of serum antibody to *Bacillus Calmette-Guérin* (BCG) were determined by solid-phase radioimmunoassays in 48 normal donors and 60 patients with bladder cancer. Of 57 patients enrolled in a randomized prospective controlled trial of BCG immunotherapy, 47 were followed for up to 30 months, thus permitting comparisons of tumor recurrence, delayed cutaneous hypersensitivity responses to purified protein derivative (PPD), and serum BCG antibody levels at specific intervals during the clinical course. Sera from normal donors and cancer patients prior to BCG therapy had equally low levels of BCG antibody. After administration of intravesical and percutaneous BCG, significant rises of serum BCG antibody levels were detected in 23 of 24 randomized BCG immunotherapy patients. Skin test responses to PPD and serum BCG antibody levels had a close correlation as immune response indicators in 14 of 24 BCG therapy patients, while rises in serum BCG antibody levels were a better response indicator than PPD skin test reactions in the other 10 patients. Eleven of the 23 patients randomized into the non-BCG treatment group had tumor recurrence, although tumors recurred in only six of the 24 randomized patients in the BCG therapy group. Two additional nonrandomized BCG-treated patients had tumor recurrence. All eight BCG-treated patients with tumor recurrence had documented increases in serum BCG antibody levels after BCG therapy. Only three of these eight patients had conversion of PPD skin test responses from negative to positive; three were positive before immunotherapy and two remained negative after BCG therapy. Levels of antibodies reactive with human adenovirus type 5 and with *Escherichia coli* antigens were similar in sera from normal donors and from the randomized bladder cancer patients in both the BCG and non-BCG treatment groups. These results suggest that serum BCG antibody responses are as useful as PPD skin tests in identifying immunological responses to the immunoadjuvant BCG during immunotherapy trials in cancer patients.

**INTRODUCTION**

During the past 10 years, various bacterial organisms and their components have been widely used as immunoadjuvants in the clinical treatment of a variety of histopathological types of naturally occurring human and animal tumors (18, 19, 24). Immune responses to the classical immunotherapeutic agent BCG have been measured in patients at intervals before, during, and after BCG immunotherapy almost universally by assessments of delayed cutaneous hypersensitivity to PPD (7). Although skin tests using recall antigens such as PPD can be useful for assessing immune competence, recent reports have suggested that serial monitoring of cancer patients using only these tests may be impractical because of subjectivity in interpretations that can result from reimmunization by repeated exposures to test antigens (5, 21).

In spite of vast amounts of information about results of delayed cutaneous hypersensitivity tests using PPD in BCG immunotherapy patients, few reports have described either quantitative measurements of humoral responses to BCG during therapy or correlations of the results of BCG antibody tests with clinical course of the BCG-treated or -vaccinated cancer patients (1, 21).

We report here current results of immune response monitoring using a newly developed solid-phase radioimmunoassay for BCG antibody in conjunction with standard PPD skin tests for delayed cutaneous hypersensitivity and correlation of these results with clinical course during a prospective randomized controlled study of BCG immunotherapy of superficial bladder carcinoma.

**MATERIALS AND METHODS**

Since January 1978, 60 patients with biopsy-proven transitional cell carcinoma have been enrolled in an ongoing prospective study of BCG immunotherapy. Forty-seven of these patients (46 males and 1 female) have, as of July 1980, been followed for up to 30 months after randomization into standard surgical therapy and standard surgical therapy plus BCG groups. Details of patient selection, randomization, BCG immunotherapy, and clinical information have been reported recently (10, 11). Briefly, patients were stratified by tumor grade (I/IV versus II/IV or more) and number of previous recurrences (0 to 1 versus 2 or more in the most recent year) and then randomized by closed envelope. BCG immunotherapy consisted of 6 weekly administrations of lyophilized Pasteur strain BCG (Institute Armand Frappier, Quebec, Quebec, Canada) using 120 mg in 50 ml 0.9% NaCl solution intravesically and 5 mg percutaneously into the upper thigh. Patients in each group were followed with cystoscopic examinations at 3-month intervals, and recurrent tumors were resected and examined microscopically. Blood specimens were obtained from 48 normal donors and from 60 bladder cancer patients at the time of their first visit. In addition, 47 of the bladder cancer patients on a randomized BCG therapy protocol provided blood specimens periodically according to a fixed schedule throughout their clinical course. Two or more blood samples were collected at least 3 months apart from 48 normal donors. Serum from each blood specimen was aliquoted into 1.0-ml portions, numerically blind coded, and then stored frozen at −50° until tested.

Coded sera were assayed for the presence of detectable...
Antibody Responses in BCG Immunotherapy Patients

Effects of BCG therapy on serum levels of BCG, Ad5, and E. coli antibody in BCG-treated and non-BCG-treated bladder cancer patients and normal donors

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>No./group</th>
<th>Treatment</th>
<th>Mean level of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BCG</td>
<td>Ad5 antibody</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Bladder cancer patients</td>
<td>24</td>
<td>BCG</td>
<td></td>
</tr>
<tr>
<td>Randomized</td>
<td>23</td>
<td>Non-BCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Normal donors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

* All patients in randomized groups were repeatedly skin tested with PPD.
* Mean BCG, Ad5, and E. coli antibody levels ± S.E. detected by SBRIA tests in sera from all subjects in each group. One pretreatment serum and 2 sera collected serially 6 to 9 months after initiation of treatment were tested from each cancer patient in the randomized groups. One original and 2 additional serum specimens collected at 6-month or greater intervals from nonrandomized cancer patients and normal donors were tested. Each serum was assayed twice in separate SBRIA tests.
+ NA, not assayed.

ABC antibody to BCG, *Escherichia coli*, and Ad5 by SBRIA methods described recently (8, 22, 25). The level of reactivity between standardized BCG, *E. coli*, and Ad5 antigen preparations and both antibody-positive and antibody-negative reference sera appropriate for each antigen was first determined by SBRIA (20, 22, 23, 25). In SBRIA tests for BCG-, *E. coli-,* and Ad5-specific antibodies, plastic-coated metal beads were sequentially reacted with (a) a preselected dilution of a standardized antigen, (b) 1% fetal bovine serum, (c) 0.5-log dilutions of reference serum or donor patient serum (primary antibody), and (d) 125I-labeled antispecies serum (secondary antibody). Beads were incubated with antigen at 38° for 1 hr, primary antibody at 4° for 18 hr, and finally with 125I-labeled secondary antibody, i.e., IgG fraction of goat anti-human IgG (Meloy Laboratories, Springfield, Va.), at 38° for 3 hr. Each bead was washed 20 times after the respective fetal bovine serum, primary antibody, and secondary antibody reaction steps while attached to a magnetic transfer device.

Results of SBRIA tests were calculated as follows. Corrected cpm were obtained by subtracting the background cpm of the diluent control from the mean cpm obtained from duplicate tests of each cancer patient and each normal donor serum versus each antigen. The relative antibody activity of each test was expressed as 125I cpm for each specific antigen. Positive BCG antibody levels in sera were indicated by mean cpm exceeding 2 times mean cpm of the BCG antibody-negative reference human serum at the same dilution (24). Positive Ad5 antibody reactivity was defined as mean cpm of test sera having 3 times or greater mean cpm of the Ad5 antibody-negative reference serum at the same dilution (25). Positive *E. coli* antibody reactions in test sera were 2 times the mean cpm of the *E. coli* antibody-negative reference serum (20).

BCG used in immunotherapy was the source of BCG antigen in SBRIA tests. BCG obtained from Institute Armand Frappier was diluted from a stock concentration of 40 mg/ml to 1:50 to 1:500 with calcium- and magnesium-free phosphate-buffered NaCl solution (0.15 M; pH 7.2) used as a diluent in all SBRIA tests. Ad5 antigens used in SBRIA tests were prepared as described previously (8, 22). *E. coli* antigens used in SBRIA tests were prepared from a stock culture of *E. coli* 014:K7:NM (CDC No. SV4411-41; Atlanta, Ga.) as described by Sanford et al. (20). Skin tests using PPD were performed in all patients prior to onset of BCG therapy and periodically throughout their course of treatment. Five units of intermediate-strength PPD in 0.1 ml were injected intradermally. Induration at 48 hr was measured and graded from 0 to 100 where the value represented the average diameter (mm) of induration measured in 2 crisscross directions.

Data from each set of SBRIA assays in the form of mean cpm ± S.E. were entered into a computer at the computer resource center at our institution, and statistical methods were then used after appropriate transformation of these data. SPSS (Statistical Package for the Social Sciences) subprogram ANOVA was used for N-way analysis of variance with up to 5 factors adjusted for up to 5 covariates, while subprogram SNK (Student-Newman-Keuls), a one-way a posteriori contrast test, was used to detect significant differences at the p = 0.05 level between all possible pairs of test groups (22, 23, 25).

RESULTS

Levels of BCG antibody in sera from the 60 bladder cancer patients before the administration of BCG therapy were not significantly different from mean BCG antibody levels detected in sera from 48 normal donors (Table 1). Significant (p = 0.002) elevations in BCG antibody levels were observed in sera from 23 of 24 randomized bladder cancer patients following BCG therapy and PPD skin tests, while sera from 4 of 23 randomized bladder cancer patients in the non-BCG treatment group showed slightly increased BCG antibody levels during follow-up after repeated PPD skin tests (Table 1).

Before BCG treatment, there was no significant difference (p = 0.68) in distribution by BCG antibody level between randomized bladder cancer patients in the BCG plus skin test group (24 patients) and in the skin test only group (23 patients). However, markedly different distributions of BCG antibody levels in patients in these 2 groups were observed after BCG therapy and repeated skin tests (Chart 1).

BCG antibody levels increased in the sera of BCG therapy patients as soon as 2 weeks after completion of BCG therapy. Peak BCG antibody levels were achieved at an average of 2.0 months for 8 patients with recurrence of tumor after BCG therapy and at an average of 4.0 months for 16 BCG-treated patients with no evidence of tumor recurrence. Highest mean

Table 1

<table>
<thead>
<tr>
<th>Subject groups</th>
<th>No./group</th>
<th>Treatment</th>
<th>BCG antibody</th>
<th>Ad5 antibody</th>
<th>E. coli antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Bladder cancer patients</td>
<td>24</td>
<td>BCG</td>
<td>1976 ± 142</td>
<td>4076 ± 116</td>
<td>1668 ± 83</td>
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<tr>
<td>Randomized</td>
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<td>Non-BCG</td>
<td>1873 ± 111</td>
<td>2400 ± 158</td>
<td>1760 ± 70</td>
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<tr>
<td></td>
<td>13</td>
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<td>2438 ± 130</td>
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<td></td>
<td>48</td>
<td>None</td>
<td>2068 ± 140</td>
<td>2155 ± 151</td>
<td>1725 ± 45</td>
</tr>
</tbody>
</table>

* All patients in randomized groups were repeatedly skin tested with PPD.
* Mean BCG, Ad5, and *E. coli* antibody levels ± S.E. detected by SBRIA tests in sera from all subjects in each group. One pretreatment serum and 2 sera collected serially 6 to 9 months after initiation of treatment were tested from each cancer patient in the randomized groups. One original and 2 additional serum specimens collected at 6-month or greater intervals from nonrandomized cancer patients and normal donors were tested. Each serum was assayed twice in separate SBRIA tests.
+ NA, not assayed.

JULY 1981

2673

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serum BCG antibody levels for all patients treated with BCG were detected at approximately 3.3 months after the completion of therapy (Chart 2A). The average time from completion of BCG therapy to a marked decrease in serum BCG antibody levels in 5 of 8 BCG-treated patients with tumor recurrence was 10.6 months, while BCG antibody levels markedly declined in sera from 13 of 16 tumor-free BCG-treated patients at 10.3 months (Chart 2B).

Comparisons of serum BCG antibody levels of both BCG and non-BCG-treated randomized patients with the percentage of patients with tumor recurrences in each group revealed that the percentage of recurrence, i.e., the number of recurrences and numbers of patients with tumors recurring, markedly increased as the BCG antibody titers declined in the BCG therapy group (Chart 3). To date, 21% (6 of 29) of randomized BCG therapy patients have had tumor recurrence. The mean disease-free interval for the BCG group is 23.6 months [Kaplan-Meier test (9)], and the average time from entry in the protocol to tumor recurrence for the subgroup into recurrent tumors is 8.2 months. Forty-six% (13 of 28) of patients in the non-BCG group have had tumor recurrence with a mean disease-free interval for the group as a whole of 14.7 months and an average time to recurrence of 7.6 months for the subgroup with recurrent tumor [p = 0.034; generalized Savage test (12); 2-way].

In the group of bladder cancer patients that received BCG therapy and repeated PPD skin tests, 5 were skin test positive and 5 were skin test negative at all test intervals. Two of the 5 patients who were always negative and 3 of the 5 patients who were always positive in PPD skin tests had recurrent tumor. Of 14 randomized patients who showed conversion from PPD skin test negative to positive, one had tumor recurrence. In the randomized non-BCG-treated group of 23 patients, 7 were PPD skin test positive and 10 were negative at all test intervals. Two of 6 patients who were not treated with BCG but showed skin test conversion from negative to positive had tumor recurrences. Seven of 10 patients who were consistently skin test negative and 2 of the 7 patients who were always skin test positive in the control group had tumor recurrence.

Within the randomized group of 24 BCG-treated bladder cancer patients, in whom serial PPD skin tests and BCG antibody responses were available, both were equally good as indicators of immune responses to BCG in 14 patients (59%), while in 10 patients (41%) serum antibody levels were better immune response reference points than PPD skin tests. PPD skin tests were not found to be superior to serum BCG antibody levels in any patients as indicators of immune responses to BCG. Representative reactions of BCG therapy patients showing antibody and skin test responses as comparable indicators...
Antibody Responses in BCG Immunotherapy Patients

Chart 4. Levels of serum antibody (•) and PPD skin test responses (A) in bladder cancer patients after BCG therapy (1) which show BCG antibody levels to be equal to (A and B) and better than (C and D) skin test responses as indicators of immune responses to BCG. Skin test response values represent average diameter (mm) of induration.

and showing serum BCG antibody response as a better indicator of immune reactivity to BCG are illustrated in Chart 4, A, B, C, and D, respectively.

Tests for BCG-specific antibody in serial specimens from the 47 bladder cancer patients in the randomized groups and from 48 normal subjects revealed that BCG therapy and repeated skin tests using PPD had virtually no effect on levels of Ad5 (viral) and E. coli (bacterial) antibodies in sera of the bladder cancer patients (Table 1).

DISCUSSION

Recently reported results of prospective randomized controlled trials in patients with superficial bladder cancer (3, 10, 11) appear to confirm earlier work by Morales (16) and Morales et al. (17), which suggested that intravesical BCG is an immunoreactive agent of benefit in the treatment of recurrent superficial bladder cancer.

Using BCG as a representative immunotherapeutic agent widely used in the treatment of a broad variety of animal and human cancers (18), a need has been identified for the development of new highly sensitive tests to monitor specific BCG antibody responses to this agent during therapy as an additional quantitative indicator of immune responsiveness (21). The SBRIA tests for BCG-specific antibody in bladder cancer patients and normal donor sera described in this report were developed in response to this need and because (a) most commonly used forms of BCG administration, such as scarification, intrapleural, intravesical, and tine techniques, ultimately introduce an unknown quantity of immunogenic BCG into the desired area, (b) measurements of delayed cutaneous hypersensitivity to PPD, as commonly used in clinical practice to serially monitor immune responses in BCG-treated patients, are subject to inconsistent interpretations and difficulties in quantitation (5, 6, 21), (c) wide cross-reactions between BCG and other microorganisms capable of provoking immune responses have been reported (14, 15), and (d) antibody to mycobacteria, such as BCG, and other bacteria has been ubiquitously detected in human sera (2, 20).

At this time, our current study evaluating BCG immunotherapy in superficial bladder cancer does not permit correlation of tumor stage with skin test reactivity. However, bladder cancer patients have been reported previously to have an impairment of primary delayed hypersensitivity responses which correlated with tumor stage (4). In other studies, no correlation with stage of disease was detected when secondary responses to recall antigens such as PPD were assessed, although bladder cancer patients showed a trend toward fewer positive skin tests with increasing quantities of tumor (6).

Minden et al. (14, 15) have suggested that antitumor activities of mycobacteria might be in part the result of shared or cross-reacting BCG-tumor antigen immunological stimuli. A possible BCG antigen-bladder cancer tumor antigen cooperative immunogenic effect as described by Minden et al. (14, 15) might have occurred in the present studies since the rise of antibody titers to highest levels was observed to occur earlier in patients with tumor recurrences than in tumor-free patients in the BCG therapy group. However, isolation and purification of tumor-specific antigens and identifications of antibodies against such antigens are needed in the bladder cancer studies before any statement about shared antigens between BCG and the neoplastic cells can be made.

The initial full report which identified the need for a quantitative and noninvasive assay for measuring the antibody response to BCG as a means of monitoring BCG immunotherapy described the measurement of BCG antibody titers by complement fixation in sera from 111 normal volunteers and 83 melanoma patients (21). Results of our studies of randomized patients with superficial bladder cancer who received BCG immunotherapy and whose immune responses were measured by SBRIA and PPD skin tests were in general agreement with the results published in these earlier studies of BCG antibody titers measured by complement fixation in melanoma patients (21). For example, low titers of BCG antibody and the distribution among patients prior to BCG therapy and controls in both studies were similar. Moreover, marked increases in BCG antibody levels in patients following BCG immunotherapy were observed in 93% (37 of 40) of the melanoma patients in the previous study (21) and in 96% (23 of 24) of the presently studied bladder cancer patients.

In contrast to the observations made in the melanoma patients that BCG antibody titers persisted in BCG-treated patients who did not have tumor recurrence (21), we found in the majority of BCG-treated bladder cancer patients that BCG antibody levels dropped with time equally in patients free of recurrence as well as in those with tumor recurrence. Although the conditions of administration of BCG therapy in our study and in the melanoma study (21) were different and generally shorter time periods after immunotherapy were studied in the nonrandomized melanoma patients, our results suggest that, if cancer patients are not boosted after the time of completion of their initial BCG therapy, BCG antibody titers will decrease in a similar way in patients with or without tumor recurrence.
Studies by Ashikawa et al. (1) showed that BCG vaccination of tuberculin skin test-negative breast, colon, and gastric cancer patients resulted in a conversion of tuberculin positivity in 17% of the patients, while 75% of these patients showed increased BCG antibody levels as measured by phosphate kaolin agglutination assays. In contrast, our results showed that BCG immunotherapy of PPD skin test-negative bladder cancer patients resulted in a PPD skin test conversion in 74% (14 of 19) of the patients and significant increases in BCG antibody levels in all of these patients. The larger numbers of patients with both PPD skin test conversion and increased BCG antibody titers in our studies can be explained in part by the 6-week BCG immunotherapy program administered to each bladder cancer patient in contrast to a single intradermal BCG injection delivered to the patients in the study of Ashikawa et al. (1).

Recent studies from prospective double-blind randomized trials for evaluating the effects of intrapleural BCG in patients with resectable lung cancer support the hypothesis of Wright et al. (13, 26, 27) that “failure to manifest PPD conversion following administration of intrapleural BCG is associated with a significantly greater risk of tumor recurrence.” Our data lend further support to this observation, but it appears that not all of the patients who benefit from BCG immunotherapy manifest PPD skin test conversion. Since skin test responses are in part subjective and are often variable on repeated testing in the same patients, the reproducibility and objectivity of SBRIA for measuring BCG-specific antibody responses make it a more effective means of monitoring an immune response to BCG. Our data suggest that decreasing antibody response to BCG parallels an increased risk of tumor recurrence in BCG immunotherapy patients.

REFERENCES


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